FUNGAL ENDOSYMBIONTS OF GRASSES, AND THEIR EFFECTS ON MULTITROPHIC INTERACTIONS

DISSERTATION

ZUR

ERLANGUNG DER NATURWISSENSCHAFTLICHEN DOKTORWÜRDE

(DR. SC. NAT.)

VORGELEGT DER

MATHEMATISCH-NATURWISSENSCHAFTLICHEN FAKULTÄT

DER

UNIVERSITÄT ZÜRICH

VON

SIMONE A. HÄRRI

AUS

BIRRWIL AG

PROMOTIONSKOMITEE

PROF. DR. CHRISTINE B. MÜLLER (VORSITZ)

PROF. DR. STANLEY H. FAETH

PROF. DR. H. CHARLES J. GODFRAY

PROF. DR. ANDREW HECTOR

PROF. DR. ADRIAN LEUCHTMANN

ZÜRICH 2007

Die vorliegende Arbeit wurde von der Mathematisch-naturwissenschaftlichen Fakultät der Universität Zürich im Wintersemester 2007/2008 als Dissertation angenommen

TO MY MUM

“Ihr Lächeln war von der echten Art. Es geschah weniger mit den Lippen als mit den Augen; das ganze Gesicht, Stirn und Wangen glänzten innig mit, und es sah aus wie ein tiefes Verstehen und Liebhaben.”

HERMANN HESSE

AND TO CLIMBING

“The combination of controlling every position of my body and of forcing my mind to shut out the ever-present urge to submit to the very real fear of falling created an interesting result: a feeling that I was simultaneously acutely aware of both everything and nothing.”

LYNN HILL

CONTENTS

GENERAL INTRODUCTION

"The scientist does not study nature because it is useful; he studies it because he delights in it, and he delights in it because it is beautiful.
If nature were not beautiful, it would not be worth knowing, and if nature were not worth knowing, life would not be worth living."

JULES HENRI POINCARÉ

GENERAL INTRODUCTION

"Clearly it is the underlying architecture, not just the parts by themselves, that maintains the bodily functions necessary for life. Analogously, the network of interactions between organisms, not diversity per se, breathes life into ecosystems. To understand the implications of biodiversity loss, it is crucial to monitor changes to the underlying 'biostructure'"

MCCANN 2007

Ecological studies classically focused on pair-wise interactions, such as those between one predator and one prey species (Lotka 1932). However, as early as 1975, when Whittaker defined a community as "an assemblage of populations of plants, animals, bacteria and fungi that live in an environment and interact with one other, forming together a distinctive living system with its own composition, structure, environmental relations, development, and function", it was recognised that all species within a community might interact in one way or another. Further, in the debate about species loss and its consequences on ecosystem functioning, it is important to focus not only on biodiversity itself but also on the underlying structuring interactions (McCann 2007). Without understanding the biological structure of ecosystems, the effects of species loss and the impact of this loss on the rest of the community is impossible to estimate.

Trophic levels are a structural characteristic of such communities of species. Species of the same trophic level are characterised by their similar means of acquiring energy (Morin 1999). The following trophic levels are commonly identified: primary producers, herbivores, primary carnivores (which feed on herbivores) and secondary carnivores (which feed on the primary carnivores). Complex interactions involving more than two trophic levels are referred to as multi-trophic level interactions and have received increasing scientific attention in recent years (e.g. Price *et al.* 1980; Müller & Godfray 1999; Schmitz, Hambäck & Beckerman 2000; Tscharntke & Hawkins 2002). Using a simple multi-trophic level model system addresses the complexity of whole food webs much more realistically than investigations of simple pair-wise interactions, but these food web modules remain simple enough to study the mechanisms involved. Interactions between several different species at different trophic levels may not only be direct, but also indirect via a third species (Wootton & Power 1993; Abrams 1995). For example, the presence of a shared predator can mediate the interaction between herbivore species feeding on different plants (apparent competition; Holt 1977). Alternatively, along a food chain, changes in the quantity of the basal resource may decrease the number of herbivores and therefore also indirectly reduce the number of natural enemies (trophic

cascades; Strauss 1991). However, indirect effects in particular are often difficult to detect in natural systems and impossible to prove without experimentation. By using a model system involving the presence of a fungal endosymbiont, I was able to manipulate the quality of a basal food resource and study its direct and indirect consequences for higher trophic levels.

Endophytic fungi

"The lush green vegetation of moist tropical forest is not what it appears. Dissolve away all the plant matter from the dense foliage, giant buttressed trunks, tangled lianas, and sinuous roots, and a ghostly fungal shadow of the forest will remain"
GILBERT & STRONG 2007

Fungal endosymbionts are not only a useful tool to study cascading effects through food chains but are an interesting phenomenon *per se* and an important structural component of natural communities and agricultural systems (Siegel, Latch & Johnson 1987; Hunter & Price 1992; Omacini *et al.* 2001). Fungi that live at least part of their life cycle asymptomatically and intercellularly within plant tissue are generally referred to as endophytes (Clay 1990; Wilson 1995). Such endophytic fungi associate with nearly all plants (Gilbert & Strong 2007). For example, leaves of tropical trees are biodiversity hotspots of endophytes (Arnold *et al.* 2000; Arnold & Lutzoni 2007). Endophytic fungi can be systemic and vertically transmitted or non-systemic and horizontally transmitted (Clay 1990). In this thesis, I focus on the systemic, vertically transmitted endophytes of the genus *Neotyphodium* (Ascomycota, Hypocreales, Clavicipitacea), which infect grasses of the subfamily Pooidae (Clay 1990). Infection by the *Neotyphodium* type endophyte results in the production of herbivore toxic compounds (Siegel *et al.* 1987). These herbivore toxic alkaloids belong to different classes, some of which are only toxic to vertebrate herbivores and others only to insect herbivores (Bush, Wilkinson & Schardl 1997).

The discovery in the 1970s that some of these alkaloids are toxic to livestock marked the beginning of increasing recognition of the presence and importance of endophytes (Bacon *et al.* 1977). The two most commonly used grass species in pastures and turf, *Lolium arundinaceum* and *L. perenne*, are both susceptible to infection by *Neotyphodium* type endophytes. Livestock feeding on endophyte-infected *L. arundinaceum* suffer from "fescue toxicosis" resulting in reduced weight gain, reduced reproduction and reduced milk production and "fescue foot", causing lameness and

soreness of the feet (Read & Camp 1986; Ball, Pedersen & Lacefield 1993). Livestock feeding on endophyte-infected *L. perenne* suffer from "ryegrass staggers", which causes muscle spasms and loss of motoric coordination (Siegel, Latch & Johnson 1985). In the US and New Zealand in particular, *Neotyphodium* type endophytes have caused large economic losses in agriculture. Consequently, recent research has focused on designing new endophyte cultivars that are toxic to insect pests but not to livestock (Easton & Fletcher 2007). This direction in agricultural research provided me with seeds from endophyte-free and endophyte-infected *L. perenne* both belonging to the same cultivar (Samson). Thus, the observed differences result only from endophyte presence but not from differences in genotypes between infected and non-infected grasses.

The presence of the *Neotyphodium* type endophyte has been shown to have mostly negative effects on herbivorous insects (Breen 1994; Popay *et al.* 2003; Hunt & Newman 2005). Therefore, the nature of the symbiosis between endophyte and grasses was assumed to be purely mutualistic with the fungus gaining nutrients and shelter and the plant receiving protection from herbivores (Clay 1988). From an evolutionary perspective, a purely mutualistic symbiosis that increases the fitness of the host plant should increase over time (Clay & Schardl 2002; Saikkonen, Ion & Gyllenberg 2002). However, in natural grasslands in Europe, endophyte infection is highly variable and rarely reaches 100% (Saikkonen *et al.* 2000; Zabalgogeazcoa *et al.* 2003). This might be explained by the fact that the effects of endophytes on communities are not as straightforward as was assumed in earlier years. Several abiotic and biotic factors, such as temperature (Salminen *et al.* 2005), mowing frequency (Salminen & Grewal 2002), water stress (Bultman & Bell 2003) and genotype interactions (Hunt & Newman 2005; Meister *et al.* 2006) have been shown to influence the impact of endophytes on herbivores. Also, not all herbivore species react negatively to the presence of endophytes (Saikkonen *et al.* 2006) and the effects of endophytes have been shown to be transmitted up the food chain to affect herbivore antagonists (Bultman *et al.* 1997; Goldson *et al.* 2000; Bultman, McNeill & Goldson 2003; de Sassi, Müller & Krauss 2006). However, it remains unclear how the presence of fungal endosymbiont in the basal resource, and more generally variation in plant quality, trickles through food webs and how it mediates interactions between and within trophic levels.

Aphid – parasitoid model systems

"To a rough approximation and setting aside vertebrate chauvinism, it can be said that essentially all organisms are insects."
MAY 1988

Aphid-parasitoid communities are a good model system to study direct and indirect effects caused by a change in the basal plant resource through the presence of endophytic fungi. Aphids feed directly by plugging into the phloem sap of plants and are thus intimately affected by the host plant quality (Dixon 1998) and possibly by its infection status. The life cycle of most aphids includes a prolonged phase of parthenogentic reproduction during summer which allows clonal lines to be cultured (Dixon 1998). The short generation time and the relative immobility of individuals enable individuals and whole populations to be followed over several generations. Additionally, winged dispersal morphs are triggered by different environmental cues such as crowding, bad plant quality and by the presence of predators (Sloggett & Weisser 2002; Kunert *et al.* 2005). Parasitoids are also intimately linked to their aphid host's metabolism as their entire larval development occurs within the body of their aphid hosts (Godfray 1994) with potentially close contact to accumulating toxins in host tissue and haemolymph. Secondary parasitoids are very diverse in aphid systems and play an important role in the regulation of communities (Müller *et al.* 1999). Aphid-parasitoid systems have been intensively studied and are well understood due to their applied importance in pest control (Brewer & Elliott 2004; Schmidt *et al.* 2004) and because parasitoids are ideal study organisms in population dynamics and theoretical models (Hassell 2000). With this model system, it is possible to create small microcosms of food web modules with varying degrees of complexity, which allows population dynamics and individual performance of species to be studied.

Goal of the project

"Earth's real biodiversity is invisible, whether we like it or not."
NEE 2004

It is known that endophytes can have community-wide effects on the structure (Omacini *et al.* 2001) and functioning (Rudgers, Koslow & Clay 2004) of ecosystems. However, the detailed mechanisms of this impact are still relatively unclear, in particular the effects of endophytes on the performance of individuals at higher trophic levels, such as primary

and secondary parasitoids. Such cascading effects to higher trophic levels mediated by plant quality are an important issue in questions related to community regulation (Chapter 1, 2 & 3). The observed reduction of aphid numbers on endophyte-infected plants in the field (Omacini *et al.* 2001) has been questioned using the argument that winged dispersal morphs might be more common on endophyte-infected plants (Chapter 4). Additionally, studies on endophytes and their role in structuring communities by affecting the performance of single species have focused only on short-term effects thus far. However, herbivores and especially those with short lifespan but fast reproduction such as aphids are likely to show adaptations to the presence of endophytes (Chapter 6). One potential adaptation might be a change in clonal composition on endophyte-infected plants. Therefore, we tested for clonal variation in the ability to cope with endophyte presence (Chapter 5).

In grass–aphid communities, one often finds several aphid species feeding on the same resource plant simultaneously. As endophyte effects are species-specific, the presence of endophytes might also regulate the competition between herbivore species and interplay with the presence of parasitoids in this regulation (Chapter 7 & 8). In the field, herbivores are regulated by their natural enemies (Hairston, Smith & Slobodkin 1960) and by resource quality and quantity (Ohgushi & Sawada 1985; Hunter & Price 1992). However, the relative importance of each of these factors is still questioned and debated. The presence of endophytes changes resource quality for herbivores and possibly their natural enemies and might therefore interplay with the regulation by natural enemies (Chapter 9). It is not only the presence of endophytes which changes plant quality but also nutrient availability, such as nitrogen. Endophyte presence and increased nitrogen availability may interact in structuring communities of aphids and their parasitoids (Chapter 10).

The presence of ubiquitous microorganisms has potentially large and complex effects on consumer interaction webs. However, the impact of these microscopic organisms on community structure and stability is still unclear. With my thesis, I try to shed some light on the interactions between well-known players of aphid-parasitoid communities and the mysterious fungal endosymbionts.

All the chapters are presented in manuscript format and already published chapters are presented in their final published version.

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SUMMARY

ZUSAMMENFASSUNG

“But what, if anything, are these microbes doing?”

KEITH CLAY

SUMMARY

The general topic of my thesis concerns the world of invisible microorganisms or, more specifically, that of endophytic fungi. Endophytic fungi live asymptomatically within the tissues of many plant species. In association with cool-season grasses, these fungi produce herbivore toxic alkaloids. Originally, this symbiosis was assumed to be truly mutualistic, with the fungus obtaining nutrients and shelter from the plant whereas the plant gains protection from herbivores. This concept of a mutualistic relationship was mainly based on studies focusing on the performance of single herbivore species in the presence of endophytes. Natural systems, however, consist of more than plants and herbivores. Thus, including higher trophic levels and using a multitrophic level approach allows us to estimate the effects endophytes may have on complex consumer interaction webs of natural systems. Within multi-species interactions, there are not only direct but also indirect effects to consider. To advance knowledge of the mechanisms by which endophytes and, more generally, variation in plant quality, influence multitrophic interactions and thus community interactions and structure, I investigated in detail the impacts of the interplay of these microorganisms with species at higher trophic levels in an aphid-parasitoid model system.

In the first three chapters of my thesis, I focused on the effects of endophytes on simple food chains in the laboratory to understand how far up the food chain the effects of endophyte-produced toxins are visible. **Chapter 1** shows that the presence of endophytes impairs the reproductive ability of an aphid primary parasitoid species without affecting its herbivore host. Interestingly, the detrimental effects of endophytes were not visible in the attack rates of endophyte-naïve females but only in the reproductive performance of individuals developing within mummies from the endophyte-infected environment. The host aphid profits from the presence of the endophytic fungi in the basal resource because it may use its toxins as defence against its natural enemy. Therefore, the endophyte may also represent a disadvantage to its plant host because the indirect positive effects of natural enemies on plant performance are disrupted. In **Chapter 2**, I confirmed that the negative impact of endophytes on primary parasitoids is not visible for endophyte-naïve primary parasitoids as they produce the same number of offspring on endophyte-free and endophyte-infected plants. Additionally, I found that the developmental time of primary parasitoids is increased on endophyte-infected plants when conditions are stressful for the aphid hosts. Increased developmental time lengthens the window for primary parasitoids to be vulnerable to attacks by secondary parasitoids. This shows that effects of

endophytes on primary parasitoids depend on environmental conditions and are amplified under conditions stressful for the associated herbivores. **Chapter 3** focuses on the next trophic level, secondary parasitoids. In a laboratory choice experiment, I offered aphid mummies from endophyte-free and endophyte-infected environments to secondary parasitoids and asked how the endophyte in the basal resource affects the life–history traits of the emerging offspring of this fourth trophic level. This experiment showed that secondary parasitoids emerging from endophyte-infected environments have a reduced lifespan and thus a reduced fitness compared to those which experienced an endophyte-free environment during larval development. Furthermore, the older and more experienced female secondary parasitoids were able to discriminate against the endophyte environment consisting of low quality hosts and adjusted their oviposition strategy accordingly.

For the following three chapters, I was interested in more specific and longer term dynamic effects of endophytes on herbivore populations. Questions address the interplay of endophytes and predators on inducible defences of aphids, clonal variation in life–history traits of aphids when exposed to endophytes and the ability of aphids to cope with the presence of the mycotoxins. **Chapter 4** was done in collaboration with Tobias Züst, an undergraduate Biology student whom I supervised. We studied the interplay of endophytes and predators on the formation of wings, a well-known inducible defence of aphids against predators. Wing induction occurred in the presence of a predator threat but was simultaneously reduced in the presence of endophytes. This result demonstrates that aphids in stressed conditions and with reduced fitness will only invest in this defence when predators are present but are unable to produce winged morphs in response to endophyte presence. **Chapter 5** takes into account the genetic background of aphids and tests for clonal variation in life–history traits of aphids when exposed to endophytic fungi in their food plant. The experiment was carried out with Atlant Bieri whom I supervised during his Master thesis. We found an interaction between aphid clone identity and presence of endophytes for some life–history traits, indicating that different clones react differently to the presence of endophytes. Thus, the gene × environment interaction plays an important role in the responses to endophyte presence and can explain the variation in tolerance to endophytes by some aphids. In **Chapter 6**, I investigated the potential of aphids to adapt to fungal derived toxins by comparing life–history traits of aphids conditioned for several generations on endophyte-infected plants with aphids conditioned on endophyte-free plants. I observed that the conditioning environment changes aphid

life–history traits whereas the endophyte infection of the test environment had a negligible effect on these aphid life–history traits. Thus, incorporating evolutionary aspects and genetics into the classical ecological research on endophytes is a promising avenue for future experiments, not only for endophyte effects on herbivores but also for effects on natural enemies.

As a next step, I introduced more complexity to the model system by adding first a second herbivore species and secondly a generalist parasitoid species. This enabled me to focus on possible indirect effects of endophytes and parasitoids on competition and coexistence between the two herbivore species in different environments. In **Chapter 7**, I asked whether endophytes can mediate coexistence of two aphid species on the same food plant. In a laboratory experiment, I showed that, for the two focal aphid species, a trade-off between tolerance to endophyte-derived mycotoxins and their competitive ability with the other species of aphid may explain herbivore coexistence on the naturally occurring stands that consist of infected and uninfected plants. In **Chapter 8**, I added a parasitoid species to the system of the two herbivore species. Parasitoids are assumed to indirectly mediate interactions between herbivore species through apparent competition. With my experimental set-up, I tested whether endophytes and parasitoids interact in influencing the co-occurrence of two herbivore species on the same species of grass. The herbivore species that was more sensitive to the endophyte was also more resistant to the parasitoids. Thus, the addition of a parasitoid species to the system enhanced the effects of endophytes and appears to lead to divergence into two different feeding niches for the two species, one on infected and one on uninfected plants. This represents a novel mechanism explaining coexistence of two competing aphid species.

As a last step, two field experiments were conducted. Firstly, I manipulated simultaneously the presence of predators and that of endophytes to determine the relative importance of these two structuring forces on natural herbivore densities in the field. Secondly, the presence of endophytes, genetic background of the plants and nutrient availability were manipulated to test the interplay of these factors on herbivore and predator abundance and species composition. In **Chapter 9,** I studied the relative importance of endophytic fungi *versus* the presence of predators in regulating population densities and dynamics of aphid herbivores in the field. By manipulating predator presence and endophytic fungi presence simultaneously, I demonstrated that the presence of predators had a strong top-down effect, whereas the presence of endophytes had only a negligible bottom-up effect. Therefore fast acting natural enemies have a stronger

regulating effect on aphid population than the presence of slower acting endophytes. **Chapter 10** shows the result of the second field experiment, in which endophyte presence, fertilizer addition and genetic background of plants were manipulated simultaneously. Fertilizer addition had a strong positive effect on plant biomass, aphid and parasitoid abundance whereas endophyte presence had no effect on insect abundances and on plant biomass. This lack of an endophyte effect in the field suggests that the crucial interactions determining aphid population growth may be more complex than those observed in simple laboratory communities and are dependent on the exact field conditions.

In conclusion, variation in plant quality caused by the presence of fungal endosymbionts triggers bottom-up cascades with measurable effects up to the fourth trophic level and with species-specific effects on the herbivores varying strongly depending on gene × environment and gene × gene interactions. The presence of fungal endosymbionts mediates herbivore species coexistence and reduces induced defence of herbivores. However, in the field, where the complexity of species interactions is considerably higher than in the laboratory experiments on simplified multitrophic systems, the slow acting endophytes do not appear to have equally strong impacts on herbivore control. The presence of endophytes modifies species interactions in a complex way and, depending on several biotic and abiotic factors, the relationship between fungi and plants can shift from mutualistic to parasitic.

ZUSAMMENFASSUNG

Gegenstand meiner Dissertation ist die Welt unsichtbarer Mikroorganismen oder spezifischer, die Welt endophytischer Pilze. Endophytische Pilze leben zwischen den Zellen vieler Pflanzenarten, jedoch ohne dass die Pflanzen Symptome zeigen. In Verbindung mit Gräsern produzieren diese Pilze Alkaloide, Substanzen, die auf Herbivoren, also Pflanzenfresser, toxisch wirken. Ursprünglich wurde angenommen, dass diese Symbiose vollkommen mutualistisch ist, da der Pilz von der Pflanze Nahrung und Schutz bekommt und im Gegenzug die Pflanze vor Herbivorie schützt. Dieses Konzept einer mutualistischen Beziehung begründete sich hauptsächlich auf Studien, die nur einzelne Herbivorenarten auf ihre Performance in Gegenwart des endophytischen Pilzes in Betracht zogen. Aber natürliche Systeme bestehen nicht nur aus Pflanzen und Herbivoren. Darum ist es wichtig, auch höhere trophische Ebenen in Untersuchungen einzubeziehen. Der multi-trophische Ansatz erlaubt es, die Effekte von Endophyten auf komplexe Nahrungsnetze genauer zu bestimmen. Innerhalb multi-trophischer Systeme gibt es nicht nur direkte, sondern auch indirekte Interaktionen. Um besser zu verstehen, wie Endophyten und allgemein Veränderungen in der Pflanzenqualität, multi-trophische Interaktionen und die Struktur von Nahrungsnetzen beeinflussen, studierte ich im Detail den Einfluss dieser Mikroorganismen und das Zusammenspiel mit höheren trophischen Ebenen in einem Blattlaus-Parasitoiden Modellsystem.

Die ersten drei Kapitel meiner Dissertation behandeln die Effekte von Endophyten in einfachen Nahrungsketten im Labor. Ziel war es, herauszufinden, wie weit nach oben in der Nahrungskette diese Effekte sichtbar sind. **Kapitel 1** zeigt, dass die Anwesenheit von Endophyten die Fortpflanzungsfähigkeit von Primärparasitoiden, so genannten Schlupfwespen, beeinträchtigt, ohne aber den Wirt, die Blattlaus, zu schädigen. Interessanterweise ist der schädliche Effekt der Endophyten nicht sichtbar in der Parasitierungsrate von Parasitoiden, die noch nie zuvor mit Endophyten Kontakt hatten. Der schädliche Effekt ist nur sichtbar bei Individuen, die sich innerhalb eines Wirtes entwickelt haben, der sich auf endophyten-infizierten Pflanzen ernährt hat. Der Blattlauswirt profitiert von der Anwesenheit des Endophyten, da er die Giftstoffe als Schutz gegen seine Prädatoren, also Räuber, brauchen kann. In diesem Fall ist die Anwesenheit der Endophyten ein Nachteil für die Pflanze, weil der indirekte positive Effekt der Prädatoren auf die Herbivoren zerstört ist. Das **Kapitel 2** bestätigt, dass der negative Einfluss der Endophyten nicht sichtbar ist bei naiven Parasitoiden, da endophyten-naive Parasitoiden unabhängig von der Anwesenheit von Endophyten

dieselbe Anzahl Nachkommen produzieren. Zusätzlich zeigt dieses Kapitel, dass die Entwicklungszeit von Parasitoiden sich verlängert, wenn sie sich auf Wirten von endophyten-infizierten Pflanzen entwickeln und zusätzlich unter Stress sind. Eine verlängerte Entwicklungszeit bedeutet eine Verlängerung des Risikos, von Sekundärparasitoiden attackiert zu werden. Die Effekte von Endophyten auf Primärparasitoiden hängen von den Umweltbedingungen ab und sind verstärkt, wenn die Wirte zusätzlich gestresst sind. **Kapitel 3** richtet sich auf die nächste trophische Ebene, die Sekundärparasitoiden. In einem Laborversuch wurden Blattlausmumien von endophyten-infizierten und endophyten-freien Pflanzen den Sekundärparasitoiden zur Wahl gestellt. Zusätzlich wollte ich wissen, wie sich die Anwesenheit von Endophyten auf die Lebensparameter von Sekundärparasitoiden auswirkt. Das Experiment zeigte, dass Sekundärparasitoiden, die aus der endophyten-infizierten Umgebung geschlüpft sind, eine reduzierte Lebensdauer haben und damit auch ihre Fitness reduziert ist. Vor allem ältere und erfahrene Weibchen bevorzugten Mumien von der endophyten-freien Umgebung.

Die nächsten drei Kapitel befassen sich hauptsächlich mit spezifischeren und Langzeiteffekten von Endophyten auf Herbivoren. Untersucht wurde das Zusammenspiel von Endophyten und induzierten Abwehrmechanismen von Blattläusen und klonale Unterschiede in der Fähigkeit der Blattläuse, mit der Präsenz des Endophyten umzugehen, sowie die Fähigkeit der Blattläuse, mit der Präsenz des Endophyten umzugehen, wenn sie diesem über längere Zeit ausgesetzt sind. **Kapitel 4** ist eine Kollaboration mit Tobias Züst, einem Biologiestudenten, den ich betreut habe. Wir untersuchten das Zusammenspiel von Endophyten und der Flügelbildung von Blattläusen, einem induzierten Abwehrmechanismus gegen Prädatoren. Die Induzierung von Flügelbildung wurde durch die Bedrohung eines Prädators ausgelöst, aber gleichzeitig durch die Endophyten stark unterdrückt. Das zeigt, dass Blattläuse unter Stress zwar in Abwehrmechanismen investieren, wenn eine Bedrohung durch einen Prädator präsent ist, aber nicht mit Flügelbildung auf die Anwesenheit des Pilzes reagieren können. **Kapitel 5** zieht den genetischen Hintergrund einer Blattlaus mit in Betracht und beantwortet die Frage, wie verschiedene Blattlausklone mit der Präsenz des Endophyten umgehen und wie dies in deren Lebensparametern zum Ausdruck kommt. Das Experiment wurde zusammen mit Atlant Bieri durchgeführt, den ich während seiner Masterarbeit betreut habe. Gefunden haben wir eine Interaktion zwischen Klonidentität und der Anwesenheit von Endophyten. Dies zeigt, dass einzelne Klone besser mit der Anwesenheit des Endophyten umgehen können als andere. Gen x Umwelt-Interaktionen spielen daher eine

wichtige Rolle im Umgang mit Endophyten und können die variable Toleranz einiger Blattläuse erklären. Im **Kapitel 6** untersuchte ich die Fähigkeit von Blattläusen, sich an die Gegenwart von Endophyten anzupassen, wenn sie diesen über mehrere Generationen ausgesetzt sind. Ich habe die Lebensparameter von Blattläusen, die den Endophyten über mehrere Generationen ausgesetzt waren mit denen von Blattläusen, die nicht den Endophyten ausgesetzt wurden, verglichen. Diese Konditionierung an die Endophyten hatte zur Folge, dass die Lebensparameter von Blattläusen unabhängig von der Infektion der Testumgebung verändert wurden. Diese kurzfristige Adaptation könnte ein Hinweis darauf sein, dass Blattläuse lernen, mit den Giftstoffen des Pilzes umzugehen. Darum ist es wichtig, in Zukunft evolutionäre und genetische Aspekte in die klassische ökologische Forschung einzubeziehen.

Im nächsten Schritt brachte ich mehr Komplexität ins Modellsystem, in dem ich zuerst eine zusätzliche Herbivorenart und dann eine Primärparasitoidenart hinzufügte, um mögliche indirekte Effekte von Endophyten und Parasitoiden auf die Konkurrenz und Koexistenz zweier Herbivorenarten zu untersuchen. In **Kapitel 7** stellte ich die Frage, ob Endophyten die Koexistenz von zwei Blattlausarten, die sich von derselben Pflanze ernähren, fördern können. Mit einem Laborversuch zeigte ich, dass für diese zwei Blattlausarten ein Trade-off zwischen der Toleranz gegenüber den Giftstoffen der Endophyten und ihrer Konkurrenzfähigkeit besteht. Dieser Trade-off führt dazu, dass diese beiden Arten möglicherweise in der Natur, wo endophyten-infizierte und endophyten-freie Pflanzen in einer Mischung vorkommen, koexistieren können. In **Kapitel 8** habe ich zusätzlich zu den zwei Blattlausarten eine Parasitoidenart mit einbezogen. Mit diesem Versuchsaufbau konnte ich feststellen, dass Endophyten und Parasitoiden sich gegenseitig in der Förderung der Koexistenz von Herbivoren beeinflussen. Die Blattlausart, die heftiger auf die Anwesenheit des Endophyten reagiert, ist resistent gegen die Parasitoiden. Darum verstärkt die Anwesenheit des Parasitoiden den Effekt des Endophyten und führt zu einer Aufteilung in zwei unterschiedliche Nahrungsnischen für diese zwei Blattlausarten; eine auf endophyten-freien Pflanzen und die andere auf endophyten-infizierten Pflanzen. Das ist ein neuer Mechanismus, der die Koexistenz von konkurrierenden Arten erklären kann.

Als letzter Schritt wurden zwei Feldexperimente durchgeführt. Im ersten wurden gleichzeitig die Anwesenheit von Endophyten und die Anwesenheit von Prädatoren manipuliert, um die relative Wichtigkeit dieser beiden Faktoren in der Regulation der Herbivorendichte zu eruieren. Im zweiten Feldexperiment wurden die Anwesenheit von

Endophyten, der genetische Hintergrund der Pflanzen und die Nährstoffverfügbarkeit manipuliert, um das Zusammenspiel dieser Faktoren auf Herbivoren und Prädatorendichte und auf die Artenzusammenstellung zu studieren. In **Kapitel 9** untersuchte ich die relative Wichtigkeit von Endophyten und Prädatoren für die Regulation von Herbivorendichte und Herbivorendynamik im Feld. Ich konnte demonstrieren, dass die Anwesenheit von Prädatoren eine starke „top-down" Kontrolle ausübte, wohingegen die Anwesenheit von Endophyten einen vernachlässigbaren Effekt hatte. Darum können die schnell wirkenden Prädatoren einen stärkeren Einfluss auf Herbivoren nehmen als die eher langsam wirkenden Endophyten. **Kapitel 10** zeigt, dass bei gleichzeitiger Manipulation von Endophytenanwesenheit, Nährstoffverfügbarkeit und genetischem Hintergrund von Pflanzen, hauptsächlich die Nährstoffverfügbarkeit einen Einfluss hat auf die Pflanzen und die Herbivoren. Das Fehlen eines Effektes durch die Endophyten zeigt, dass die Interaktionen, die das Blattlauswachstum im Feld beeinflussen, sehr viel komplexer sind als jene in einfachen Laborsystemen.

Zusammenfassend lässt sich sagen, dass die Veränderung in der Pflanzenqualität, ausgelöst durch die Anwesenheit eines endophytischen Pilzes, eine „bottom-up" Kaskade auslöst, deren Effekte bis zur vierten trophischen Ebene sichtbar sind. Die Effekte sind aber artenspezifisch und variieren stark abhängig von Gen x Umwelt- und Gen x Gen-Interaktionen. Die Anwesenheit eines endophytischen Pilzes fördert die Koexistenz verschiedener Herbivorenarten und reduziert die Abwehrmechanismen von Blattläusen. Aber im Feld, wo die Komplexität der Interaktionen zwischen den Arten erheblich höher ist, haben die langsam wirkenden Endophyten weniger starke Effekte auf die Kontrolle von Herbivoren. Die Anwesenheit von Endophyten modifiziert Interaktionen zwischen den Arten auf komplexe Art und Weise und ist abhängig von verschiedenen biotischen und abiotischen Faktoren. Dies führt dazu, dass sich die Beziehung zwischen Endophyten und Pflanzen von mutualistisch zu parasitisch verschieben kann.

CHAPTER 1

"I cannot persuade myself that a beneficent and omnipotent God would have designedly created the Ichneumonidae with the express intention of their feeding within the living bodies of Caterpillars."

CHARLES DARWIN

TROPHIC CASCADES INITIATED BY FUNGAL PLANT ENDOSYMBIONTS IMPAIR REPRODUCTIVE PERFORMANCE OF PARASITOIDS

SIMONE A. HÄRRI, JOCHEN KRAUSS & CHRISTINE B. MÜLLER

ABSTRACT

Variation in plant quality can transmit up the food chain and may affect herbivores and their antagonists in the same direction. Fungal endosymbionts of grasses change the resource quality by producing toxins. We used a model system of an aphid and its parasitoid to explore how endophyte effects cascade up the food chain and influence individual parasitoid performance. We show that the presence of an endophyte in the grass *Lolium perenne* affects the performance of the parasitoid *Aphidius ervi* negatively without showing clear effects on its host *Metopolophium festucae*. Although the presence of endophytes did not influence the parasitism rate of endophyte-naïve parasitoids or their offspring's survival to adulthood, most parasitoids developing within aphids from endophyte-infected plants did not reproduce at all. This indicates a delayed but very strong effect of endophytes on parasitoid performance, which should ultimately affect plant performance negatively as their herbivores are released from top-down limitations.

INTRODUCTION

Natural communities and ecosystems have a huge diversity of species, which corresponds with an enormous diversity of interactions among species. To understand the nature and magnitude of these interactions among species within whole communities it is vital to estimate the impact of species loss on ecosystem functioning (McCann 2007). Such biotic interactions can be direct, involving only two species (e. g. plant-herbivore interactions), or they can be indirect, involving more than two species (e. g. trophic cascades, apparent competition; Strauss 1991). Indirect effects are common and important for structuring natural communities but are only detectable by experimentation (Holt & Lawton 1993; Müller & Godfray 1999; Werner & Peacor 2003). Indirect effects propagating upward or downward through a food chain are called trophic cascades and may be common in many ecological communities (Carpenter, Kitchell & Hodgson 1985; Pace *et al.* 1999; Schmitz, Hambäck & Beckerman 2000). Classically, trophic cascades are defined as 'top-down'

when the removal of predators results in increased plant abundance through a reduction in herbivore numbers (Hairston, Smith & Slobodkin 1960; Pace *et al.* 1999). For example, the removal of largemouth bass, a top predator in prairie streams, leads to a decrease in algal abundance caused by an increase in the herbivorous minnows (Power, Matthews & Stewart 1985). However, cascading indirect effects can also propagate upwards or 'bottom-up'.

Bottom-up cascades are generally less well studied, but were shown to be common in terrestrial systems as a structuring force (Hunter & Price 1992; Denno *et al.* 2002). In contrast to 'top-down' cascades, most studies on 'bottom-up' cascades show directional responses that are the same for herbivores and their natural enemies, i.e. bad plant quality decreases herbivore population density as well as natural enemy performance and good plant quality enhances herbivore abundance as well as natural enemy performance (Hunter & Price 1992; Nakamura *et al.* 2005; Kagata & Ohgushi 2006). For example, Nakamura *et al.* (2005) showed that increased foliage sprouting after a flood increased the abundance of leaf beetles and their natural enemies. It has also been shown that 'bottom-up' cascades can be triggered by chemical defence compounds of plants, but here the cascading effects on natural enemies are usually weak or absent (Kagata & Ohgushi 2006; but see Soler *et al.* 2005). This interruption on cascading effects by defence compounds can be caused by herbivorous insects that are resistant to the plant's defensive toxins or by herbivores accumulating the plant secondary compounds within their own body tissue and using them for their own defence against natural enemies (Barbosa, Gross & Kemper 1991; Francis *et al.* 2001). Chemical defences are not produced exclusively by plants themselves, but also by symbiotic associations of plants with microbes, as is the case for many grasses that are badly protected by secondary plant compounds but frequently associate with systemic, seed-borne endophytic fungi that produce herbivore toxic compounds (Clay 1990; Bush, Wilkinson & Schardl 1997).

Endophytic fungi (= endophytes) live intercellularly in leaf and stem tissue (Clay 1990; Schardl, Leuchtmann & Spiering 2004). The consequences of this grass-fungus alliance for the herbivores are fairly well understood, especially for endophytes of the genus *Neotyphodium* found in association with cool-season grasses, where they reduce herbivore performance in most cases (Faeth & Bultman 2002; Hunt & Newman 2005; Meister *et al.* 2006). A few studies have shown that grass symbionts can trigger 'bottom-up' cascades and alter the performance of and the interactions among consumers and their natural enemies at an individual, population or community level (Omacini *et al.* 2001; de

Sassi, Müller & Krauss 2006; Finkes *et al.* 2006). However, experimental studies on endophytes and their effects on higher trophic levels are still rare (Faeth & Bultman 2002; Müller & Krauss 2005). The existing studies have generally found negative effects on herbivores and no or negative effects on the associated natural enemies (Barker & Addison 1996; Bultman *et al.* 1997; Goldson *et al.* 2000; Bultman, McNeill & Goldson 2003; de Sassi *et al.* 2006). However, no study so far investigated the effects of endophytes on the reproductive ability of parasitoids that developed within herbivores feeding on endophyte-infected plants. Other studies on endophytes and parasitoids mainly concentrated on the parasitism rate (= attack rate) of endophyte-naïve individuals and on developmental time and survival to adulthood of their offspring (Barker & Addison 1996; Bultman *et al.* 1997; Bultman *et al.* 2003). Here, we additionally look at fecundity traits (i.e. fitness) of parasitoids because changes in fecundity traits of individuals will ultimately determine the dynamics of whole populations. In addition, we do not restrict the study to simple parasitoid responses but follow the offspring generation through its development and its own reproductive performance. This allows for the detection of possibly delayed effects of endophytes on parasitoid fitness. The effects of endophytes on natural enemies may be *(1)* direct (fungal-derived toxins accumulate in the prey or host tissue and directly harm predators and parasitoids), *(2)* indirect via changes in herbivore densities (density-mediated), or *(3)* indirect via changes in life-histories or the behaviour of herbivores (trait-mediated; Wootton 1993; Abrams 1995; van Veen, Morris & Godfray 2006).

By manipulating endophyte presence in the basal resource, we can compare multi-trophic interactions among consumers and their enemies on grasses with and without endophytes. This approach allows studying changes in basal resources and the associated cascading effects upwards the food chain. Additionally, the grass-endophyte system is of agricultural importance as it is used to protect pastures from insect pest herbivores (Popay *et al.* 2003). Within the framework of biological control, it becomes important to understand not only direct effects of endophytes on herbivorous consumers but whether and how endophytes affect the enemies of insect pest herbivores. The aphid-parasitoid system proofs to be a good model system to test the effects of endophytes on several trophic levels. Aphids feed directly on the phloem sap of the plants and are thus intimately affected by the host plant quality (Dixon 1998) and possibly by its infection status. Parasitoids are also intimately linked to their aphid host's metabolism as their whole development occurs within the host's body (Godfray 1994) with potentially close

contact to accumulating toxins in host tissue and haemolymph. Aphid-parasitoid systems are well understood because of their applied importance in pest control (Schmidt *et al.* 2003; Brewer & Elliott 2004) and because parasitoids are ideal organisms for the empirical and theoretical study of population dynamics (Hassell 2000). Measurements of attack rates, longevity and reproductive success of parasitoids allows to make a direct link between life-history traits and expected population densities and dynamics.

Here, we tested for the effects of the endophytic fungi *Neotyphodium lolii (*Glen, Bacon and Hanlin)*,* a fungal endosymbiont of the pasture grass *Lolium perenne* (L.), on individual life-history traits of the cereal aphid *Metopolophium festucae* (Theobald) and on fitness estimates of its primary parasitoid *Aphidius ervi* (Haliday) (Braconidae: Aphidiinae). This food chain occurs naturally on wild grasses, even though the tested grass-endophyte association is of agricultural origin. We followed parasitoids over more than one generation; in the endophyte-naïve parental generation we measured differences in attack rates and in the first parasitoid offspring generation that developed either within aphids feeding on endophyte-free or on endophyte-infected grass, we tested several life-history traits including the fecundity (= lifetime reproductive success). To separate between the effects of endophytes on parasitoid fecundity and those on the oviposition decision behaviour per se, females of the first offspring generation were all offered aphids from endophyte-free plants. We hypothesised that herbivores reared on endophyte-infected grass and primary parasitoids emerging from these herbivores both have fitness disadvantages that are reflected in the life-history traits we measured. We found that while the endophyte in the grass did not clearly affect aphid performance, parasitoids developing within hosts from the endophyte-infected environment suffered from strongly reduced reproductive performance.

MATERIAL AND METHODS

Lolium perenne seeds were provided by Brian Tapper (AgResearch, NZ). All seeds were the Grassland Samson cultivar and were either uninfected (E -; identity number: 11104, < 0.01% infection) or infected with the common wildtype endophyte *N. lolii* (E +; identity number: A12038, 89% infection). The infection status of the seed batches was checked with a combination of microscopic examination of stained seeds and immunoblotting of stems (see Härri, Krauss & Müller 2007). The stock culture of *M. festucae* was started in summer 2005 with a few individuals collected from *L. perenne* near the University of Zürich, Switzerland. This aphid culture was maintained on commercially available

endophyte-free fodder grass *L. perenne* ARION (fenaco, Winterthur, Switzerland; staining of 30 seeds: 0% infection). The stock culture of *A. ervi* was started with 250 individuals bought from Andermatt Biocontrol AG, Grossdietwil, Switzerland. *Aphidius ervi* was kept on *M. festucae* feeding on *L. perenne* ARION. All insect cages and experiments were kept in controlled environment chambers at 22ºC with a L16 : D8 h light regime.

Life-histories of the aphid M. festucae

To test for the effects of *N. lolii* on individual life-history traits of *M. festucae*, single first instar nymphs were each followed for their whole life on cuttings of either E- or E+ *L. perenne*. The replication was 20 for each treatment and cuttings were exchanged every second day. The measured life-history traits were *(1)* developmental time (time from first instar nymph to a mature adult), *(2)* fecundity (total number of offspring produced during an adult's life), *(3)* daily fecundity (mean number of nymphs produced per day during the period of adult life) and *(4)* lifespan (here defined as reproductive lifespan, i. e. the number of days being adult). After death, the hind tibia length was measured for each mother to assess possible effects on body size. Tibia length measurements were also taken for the first 10 nymphs of each mother, at the age of maximally one day after birth.

Life-histories of the parasitoid A. ervi

The duration of the experiment covered three parasitoid generations: the parental generation and the first and second offspring generation. This allowed us to measure lifetime reproductive success of parasitoids and possible delayed effects on parasitoid fitness developing on aphids from E- and E+ *L. perenne*. Parasitoids of the parental generation originated from a stock culture that had no previous experience with aphids from E+ grasses. The individuals of the first parasitoid offspring generation developed either in aphids feeding on E- (E- offspring) or aphids feeding on E+ (E+ offspring). The fecundity of the E- offspring was compared with that of the E+ offspring and their progeny is the second offspring generation. All the potted plants used in the experiments were covered with an inverted PET - bottle that had two windows covered with mesh for ventilation.

Parental generation—The parental generation was collected as parasitised aphids (= mummies) from the stock culture. After emergence, females were allowed to mate for 12

hours with two males of the same age in a plastic vial sealed with a foam stopper (5 cm x 2 cm). The mating individuals were provided with a piece of apple. This procedure was repeated 40 times to obtain 20 females for each of the two endophyte treatments (E- and E+). The female together with the two males were then placed on a pot (E- or E+; ø 10 cm, 100 seeds per pot, 6-days old) with *ad libitum* number of aphids and left for 24 hours. After parasitoid removal, the pots were left for 7 days after which aphids were transferred to a fresh pot of grass of the respective treatment (E- or E+; ø 10 cm, 100 seeds per pot, 6-days old). After another 7 days, all mummies were removed and placed singly into gelatine capsules. The mummies in the gelatine capsules were checked twice a day for emergence of the first offspring generation.

The recorded life-history traits of the parental generation were *(1)* the proportion of females (mothers) producing mummies, *(2)* the number of mummies produced per mother, *(3)* the proportion of mothers producing viable offspring, *(4)* the number of offspring produced per mother and *(5)* the sex ratio of their offspring (proportion of males).

1st offspring generation—The fecundity of all female parasitoids produced by the parental generation was tested on *ad libitum* number of aphids feeding on *L. perenne* ARION pots (ø 10 cm, 100 seeds per pot, 6-days old). The parental generation produced 42 E- and 25 E+ females. These females were mated with one similar aged preferably unrelated male from the same endophyte treatment, avoiding brother-sister matings. As not for all females males of the same age and the same treatment were available for mating, only 32 E- and 15 E+ females were mated. Pairs or single females were kept for half a day in a plastic vial. Thereafter the females were transferred singly onto the pots with the aphids. After 24 hours, the female parasitoids were transferred to a fresh pot with *ad libitum M. festucae*. This was repeated every 24 hours until the female died. Most of the females died within the first 24 hours. This resulted in too little variation in the data of longevity to allow for a sensible statistical analysis of this data.

The pots with the potentially parasitised aphids were left for 7 days before transferring all aphids on a fresh pot of *L. perenne* ARION (ø 10 cm, 100 seeds, E- or E+, 5-days old). Aphids were left to form mummies for another 7 days before these mummies were collected and individually placed into gelatine capsules. The capsules were checked daily for emerged parasitoids. The sex of the emerged parasitoids was determined and they were placed into alcohol.

The fitness estimates obtained for the first offspring generation were the same as for the parental generation (*1 – 5*), but in contrast to the parental generation, these estimates are more meaningful as the individuals were followed and experienced our treatment over their entire lifespan. Therefore, the measurements on the number of viable offspring refer to total fecundity (= lifetime reproductive success). Additionally, for the first offspring generation we measured *(6)* emergence rate (= survival to adulthood) and *(7)* days to emergence (= developmental time).

2nd offspring generation—The offspring produced by the E- and E+ female parasitoids from the first offspring generation are called second offspring generation. For the second offspring generation, *(6)* survival to adulthood and *(7)* developmental time were measured as life-history traits.

Statistical analyses

All statistical analyses were performed using R (version 2.5.0 for MacOS X). All means are presented as mean ± 1 SE. Aphid life-history traits were either analysed with ANOVAs with endophyte treatment as explanatory variables or linear mixed effects model (LME) with endophyte treatment as fixed effects and mother identity as random effect. Developmental time and fecundity of aphids were ln-transformed to meet assumptions of normality and heteroscedasticity of the model residuals. Replication number differed between treatments because in one replicate the nymph did not reach adulthood and one replication was lost after the nymph reached adulthood. For the analysis of development time, replicates where the nymphs were second instars at the beginning of the experiment instead of first instar were excluded (E+: 5, E-: 2).

In all the analyses on *A. ervi*, endophyte treatment was included as a fixed factor. The measured life-history traits were either analysed with ANOVAs, linear mixed effects models (LME), including mother identity as random effect, generalised linear models (GLM) with quasibinomial error structure to correct for the overdispersion, or generalised mixed effects models (GLMMPQL) with mother identity as random effect and a quasibinomial error structure (Venables & Ripley 2002). The use of each model is indicated directly in the results section.

For the number of mummies *(2)* and the number of viable offspring *(4)* only females producing at least one mummy or one viable offspring respectively, were included into the analyses. These numbers were ln–transformed. For the analyses of *(1)* to

(5) of the first offspring generation, including the mating status (yes/no) did not explain much of the variance and was therefore neglected. The sex ratios *(5)* were only analysed for replicates where at least one female emerged, as only for these it was certain that the female had been mated. Including all replicates did not make a difference for calculating significance levels. Sex ratios were analysed only for the parental generation, as the lack of offspring produced by the first generation did not allow analysing sex ratios (see results). The sex ratios and the survival to adulthood *(6)* were arcsine-square-root-transformed. For the analyses of developmental time *(7)*, the sex of the offspring was included as a fixed effect.

RESULTS

Life-histories of the aphid M. festucae

The presence of the endophyte in the grass had no clear negative effects on *M. festucae*. The developmental time was not significantly influenced by the presence of endophytes (ANOVA: $F_{1,30} = 0.12$, $P = 0.733$; Fig. 1a). Fecundity tended to be slightly lower on E+ than on E- plants (ANOVA: $F_{1,36} = 3.87$, $P = 0.057$, Fig. 1b). However, daily fecundity (ANOVA: $F_{1,36} = 1.95$, $P = 0.172$; Fig. 1c) and lifespan (ANOVA: $F_{1,36} = 2.36$, $P = 0.133$; Fig. 1d) were not significantly affected by the presence of endophytes. The body size of the mothers (ANOVA: $F_{1,36} = 0.94$, $P = 0.340$) and the body size of the nymphs (LME: $F_{1,37} = 1.50$, $P = 0.228$) were not significantly affected by the presence of endophytes.

Life-histories of the parasitoid A. ervi

Parental generation—From the parental generation, 14 out of the 20 females on E- (70%) and 15 out of the 20 females on E+ (75%) produced at least one mummy; this proportion of females producing mummies did not differ between the endophyte treatments (GLM: $F_{1,38} = 0.12$, $P = 0.732$). Also the number of mummies resulting from each female that produced at least one mummy did not differ significantly between the endophyte treatments (ANOVA: $F_{1,27} = 0.85$, $P = 0.365$). The proportion of parental generation females producing at least one viable offspring (GLM: $F_{1,38} = 0.00$, $P = 1.00$; Fig. 2a) and the number of viable offspring produced by these females did not differ between the endophyte treatments (ANOVA: $F_{1,26} = 2.20$, $P = 0.150$; Fig. 2c). Also the sex ratio of the first offspring generation with 31.10 ± 9.42% on E- and 46.59 ± 6.04% on E+ was not significantly influenced by the presence of endophytes (ANOVA: $F_{1,26} = 2.01$, $P = 0.177$).

1st offspring generation—The survival to adulthood (proportion of emerged first offspring individuals out of the mummies produced by the parental generation) was not significantly influenced by the presence of endophytes with 66.06 ± 5.58% on E- and 52.04 ± 6.99% on E+ (ANOVA: $F_{1,27} = 2.01$, $P = 0.168$). Similarly, the developmental time of the first offspring generation was not significantly influenced by the presence of endophytes (LME: $F_{1,26} = 0.39$, $P = 0.540$), but as expected for parasitoids, developmental time was approximately one day longer for females than for males (females: 18.59 ± 0.17 days, males: 17.79 ± 0.21 days; LME: $F_{1,138} = 10.03$, $P = 0.002$).

All of the 42 E- and 25 E+ female offspring emerging in the 1st generation were tested for their fecundity. Out of these females, 40 E- females produced at least one mummy whereas only 6 E+ females produced at least one mummy (Proportion of females producing mummies, GLMMPQL: $F_{1,18} = 22.44$, $P < 0.001$). The E- females producing mummies also produced significantly more mummies than E+ females (LME: E-: 11.45 ± 1.13, E+: 5.33 ± 3.94; $F_{1,13} = 12.36$, $P = 0.004$). For the proportion of first offspring generation females producing viable offspring, this difference was even more pronounced (GLMMPQL: $F_{1,18} = 22.76$, $P < 0.001$; Fig. 2b). However, the three E+ females that had viable offspring produced a similar number as the 38 E- females (LME: $F_{1,10} = 0.84$, $P = 0.380$; Fig. 2d). The sex ratio of the offspring from the E+ females was not analysed, as only one female out of the three reproducing E+ female parasitoids was previously mated. The mated E+ female parasitoid produced 25 mummies out of which 12 were females. The sex ratio of the offspring from the E- females was 40.22 ± 5.76%.

2nd offspring generation—The survival to adulthood (LME: $F_{1,13} = 0.71$, $P = 0.413$) and developmental time (LME: $F_{1,10} = 0.001$, $P = 0.975$) were not significantly influenced by the presence of endophytes. As in the first generation, the developmental time was longer for females than for males (females: 17.76 ± 0.12 days, males: 16.79 ± 0.09 days; LME: $F_{1,239} = 42.31$, $P < 0.001$).

DISCUSSION

In our study we demonstrated that the presence of a fungal endosymbiont in the basal resource of an insect food chain reduced the reproductive performance of parasitoids even though the associated herbivore species showed no clear negative effect. The detrimental effect of the endophyte on the parasitoids was not visible in the parasitism rate of endophyte-naïve females, which suggests that they attack E+ and E- hosts equally.

However, parasitoids that developed within hosts from endophyte-infected plants had a highly impaired reproduction with only 12% of females from E+ compared to 90% from E- producing any offspring.

Even though the overall fecundity of the aphid host *M. festucae* tended to be reduced on E+ plants ($P = 0.057$), we argue that this species is rather insensitive to endophyte presence. This is mainly because none of the other measured traits showed any differences, but also because in a population level experiment the numbers of *M. festucae* were similar on E- and E+ (S. A. Härri, unpublished data). This lack of a clear negative effect on *M. festucae* is also in accordance with field studies based on the same and on a different grass-fungus association (Omacini *et al.* 2001; Krauss *et al.* 2007). This relative insensitivity of the host species towards the presence of endophytes associated with the strong detrimental effects on the parasitoids leads to an upward cascade that does not affect herbivores and their natural enemies in the same direction. A result that clearly contrasts with typical bottom-up cascades described so far, whereby plant consumer levels are affected in the same direction as herbivore consumer levels (e. g. Teder & Tammaru 2002; Soler *et al.* 2005). However, the presence of allelochemicals in host plants has been associated with weakened or interrupted trophic cascades (Kagata & Ohgushi 2006). For example, in tobacco plants with high concentrations of nicotine, little or no effect on the herbivore was detected, although the toxins caused mortality of parasitoids (Barbosa *et al.* 1991). This phenomenon is referred to as "toxic environmental effect" (Hunter 2003) but is supposed to be rare (Hunter 2003; Kagata & Ohgushi 2006). The "toxic environmental effect" shows that herbivores insensitive to certain plant allelochemicals may use these substances to defend themselves against attacks by predators and parasitoids (Campbell & Duffey 1979; Kazana *et al.* 2007).

With our experimental set-up, we were able to distinguish between detrimental effects by endophytes caused during the developmental time and those caused by differences in attack rates. Had we not studied the reproductive performance of the first generation, we would have concluded that the parasitoids show no difference in behaviour on E+ and E- hosts. By following the offspring performance, we demonstrated that parasitoids developing within herbivores feeding on endophyte-infected plants were negatively affected and most were unable to reproduce at all. The reduction in reproductive success could have had several reasons, which we were unable to identify in our experiment. It remains unknown how the mycotoxins that are produced by the symbiosis of plant and fungus can affect the reproductive success of parasitoids. It is

possible that the exposure to toxins during larval development lead to a general weakness of the adult parasitoid females. These weak E+ females may either not have been able to find or attack the aphids or they attacked the aphids successfully but the eggs may not have been protected enough to evade destruction by the aphid immune system. Herbivorous insects do not have as sophisticated immune responses as vertebrates, but they can encapsulate parasitoid eggs and thus prevent them from developing. The ability to encapsulate parasitoid eggs can vary substantially between individuals (Henter & Via 1995) and has been shown to trade-off against competitive ability in *Drosophila melanogaster* (Kraaijeveld & Godfray 1997). A reduction in the fitness of parasitoids could also be explained by a small host size (Godfray 1994). However, as the body size of *M. festucae* was not influenced by the presence of endophytes, we can exclude such an indirect endophyte effect caused by smaller sized hosts.

The few studies that investigated whether endophytic fungi can interact with natural enemies used systems where the herbivore also shows reduced performance on endophyte-infected plants (Barker & Addison 1996; Bultman *et al.* 1997; Bultman *et al.* 2003; de Sassi *et al.* 2006). Ours is the first study to show that an herbivore species that is relatively tolerant to the presence of endophytes propagates the endophyte effects to its enemy and thus receives protection from parasitoids. Ultimately, the negative effects of endophytes on parasitoids but not on herbivores should result in a release from top-down limitation by parasitoids. In combination with the absence of bottom-up limitation of herbivores by endophytes this should lead to pest outbreaks.

The lack of effect on endophyte-naïve females and the strong reduction in reproductive ability of their offspring represents a delayed effect. Such delayed life-history effects are well known as maternal effects (Beckerman *et al.* 2002), where phenotypic variation in the offspring is caused by differences in the environment experienced by the mother (Rossiter 1996; Mousseau & Fox 1998). In our system, the delayed effect results from the lacking effect of endophytes on survival to adulthood but a strong detrimental effect on the reproductive ability of these females. Possible maternal effects could have arisen when females were given the opportunity to discriminate against hosts from endophyte-infected plants. In our experiment, the initial females were restricted to either hosts from the endophyte-free or the endophyte-infected environment. Field experiments on the same grass-endophyte system did not provide any evidence for parasitoid's choices, as similar numbers of parasitoid mummies were detected on endophyte-free and endophyte-infected plants (Krauss *et al.* 2007). In this field

experiment however, mummies of different parasitoid species were not allocated to different aphid species and there might have been an undetected species-specific effect of endophytes.

In a multi-trophic setting, a plant may be protected from damage by some herbivorous species when entering an alliance with fungal endosymbionts. However, if there are species that can withstand the mycotoxin and additionally transfer toxic effects to their natural enemies, the plant pays a three-fold cost: *(1)* it provides resources for the endophyte, *(2)* it is still attacked and consumed by the particular herbivore that is tolerant to the mycotoxins and *(3)* it suffers from an indirect density-mediated effect of reduced biological control by parasitoids. It would be interesting to discover how secondary parasitoids that are exceptionally numerous in aphid system (Müller *et al.* 1999) are affected by the mycotoxins in this trophic chain.

In conclusion, we show that the presence of endophytic fungi in the agricultural pasture grass *L. perenne* transmits up the food chain without showing impaired performance of the herbivores. We detected strong negative effects of the endophyte *N. lolii* on lifetime reproductive success of primary parasitoids, although the rate of parasitism by endophyte-naïve parasitoids was not affected by the presence of the fungal endosymbiont. The nature of this microorganism - plant association has diverse, sometimes unpredictable impacts on higher trophic levels and should therefore be considered in future research on multi-trophic interactions.

ACKNOWLEDGMENTS

We thank Julia Nüscheler and Virginie Boreux for practical help in the laboratory, Brian Tapper (AgResearch, NZ) for contributing the seeds, Adrian Leuchtmann (ETHZ) for help with endophyte detection, Swiss Caps AG (Kirchberg, Switzerland) for providing the gelatine capsules and Dennis Hansen and Tom Jones for helpful discussions and comments on the manuscript. The project was funded by a SNSF grant to cbm (631-065950).

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FIGURES

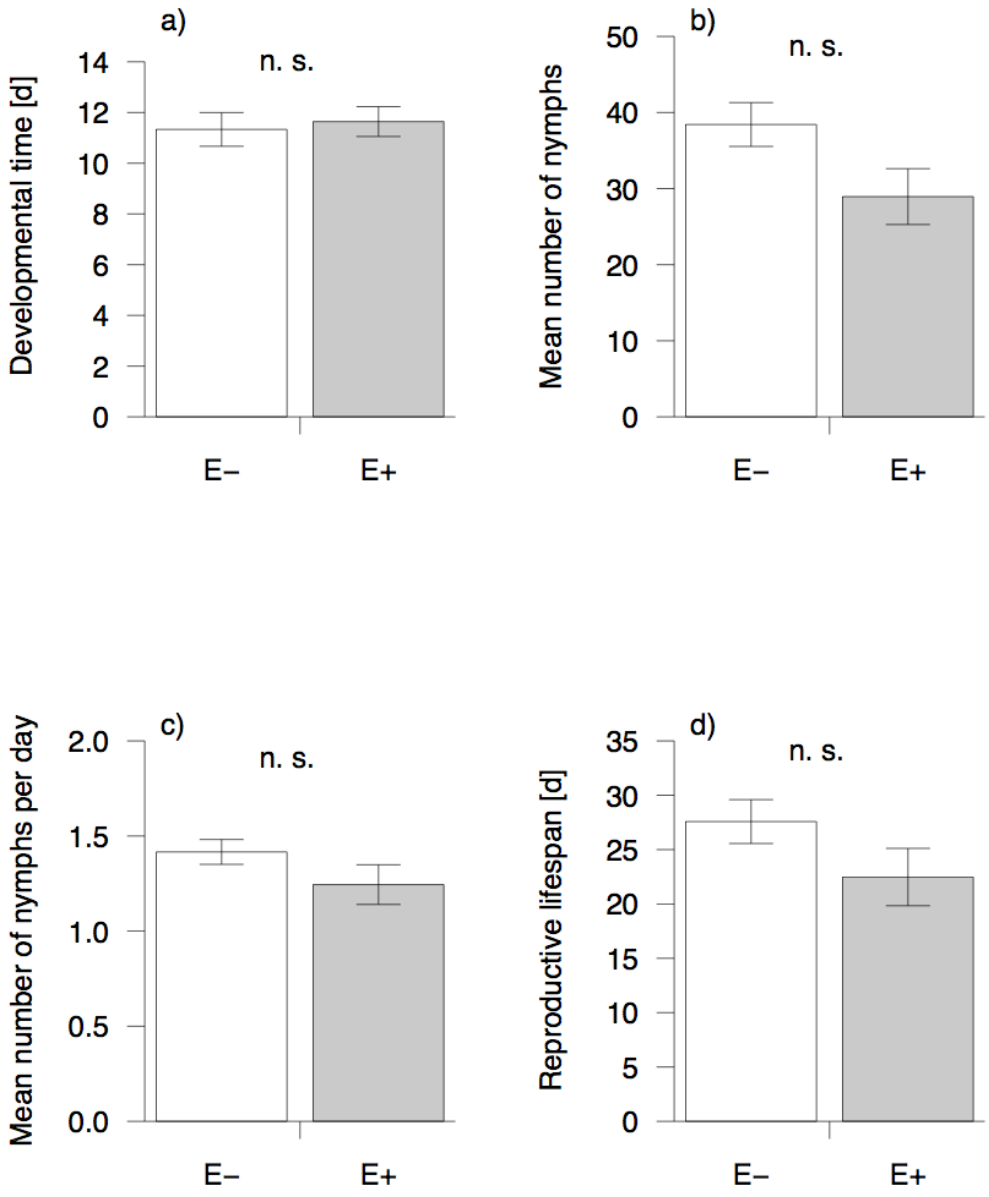

FIGURE 1. Aphid performance. Development time (*a*), fecundity (*b*), daily fecundity *(c)* and adult lifespan *(d)* for the aphid species *M. festucae* feeding on endophyte-free (E-) or endophyte-infected (E+) *L. perenne*. The error bars show ± 1 SE. None of the measured life-history traits were significantly affected by endophyte presence. However, fecundity tended to be slightly reduced on E+ ($P = 0.057$). n. s. indicates not significant ($\alpha = 0.05$).

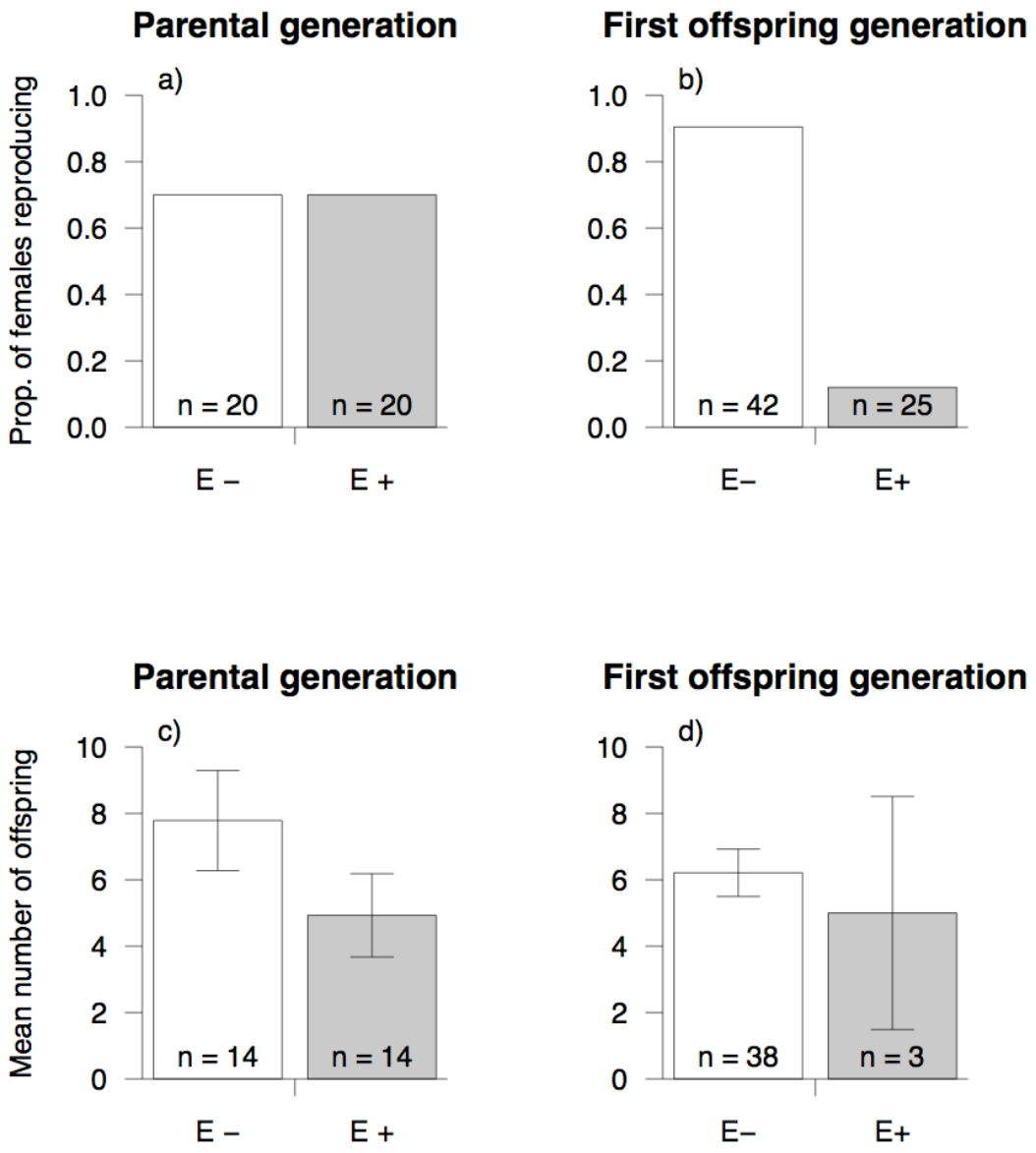

FIGURE 2. Parasitoid performance. The proportion of parasitoid females reproducing on endophyte-free (E-) or endophyte-infected (E+) *L. perenne* in a) the parental generation and b) the first offspring generation. Mean (± 1 SE) number of offspring produced by c) the parental generation and d) the first offspring generation. *n* indicates the number of replicates. For the first offspring generation, the difference in replication between E- and E+ and the start of the experiment is caused by the fact that all females produced by the parental generation were used for the test of fecundity and an unequal number of first offspring generation females were produced on E- and the E+ treatments. For the number of offspring produced (c and d) only females, which produced at least one viable offspring were included into the analyses.

CHAPTER 2

EXTENDED LARVAL DEVELOPMENT TIME FOR PRIMARY PARASITOIDS IN THE PRESENCE OF PLANT ENDOSYMBIONTS

SIMONE A. HÄRRI, JOCHEN KRAUSS & CHRISTINE B. MÜLLER

ABSTRACT

1. Variation in plant chemistry does not only mediate interactions between plants and herbivores but also interactions between herbivores and their natural enemies. Insect herbivores may use plant allelochemicals as acquired defence against their natural enemies.

2. Endophytic fungi that complete their whole life cycle within the host plant's tissue are associated with a large diversity of plant species. Endophytes of the genus *Neotyphodium* alter the chemistry of the host plant by producing herbivore toxic alkaloids.

3. Here we asked whether the endophyte-tolerant aphid species *Metopolophium festucae* can use fungal derived toxins as acquired defence against its parasitoid *Aphidius ervi*. In a laboratory experiment we compared life-history traits of *A. ervi* when exposed to hosts on endophyte-infected or endophyte-free *Lolium perenne*.

4. The presence of endophytes significantly increased larval and pupal development times, but did not affect the mortality of immature parasitoids or the longevity of the adults. Even though the number of parasitoid mummies tended to be reduced on endophyte-infected plants, the number of emerging parasitoids did not differ significantly between the two treatments.

5. This shows that individual aphids were only partly able to use the fungal derived mycotoxins in defending against parasitoids. An increase in parasitoid development time should ultimately reduce the population growth of *A. ervi*. Therefore, endophyte presence may represent an advantage for endophyte-tolerant aphid species through extended parasitoid development and its effect on parasitoid population dynamics.

INTRODUCTION

Variation in plant quality and plant chemistry can mediate interactions between herbivores and their natural enemies (Price *et al.* 1980). Generally, effects of plant allelochemicals on herbivore enemies are assumed to be positive, such as herbivore induced plant volatiles that attract natural enemies (Turlings, Tumlinson & Lewis 1990; Ode 2006). However, other studies also showed that plant allelochemicals may deter or impair the fitness of natural enemies by rendering their hosts toxic (Campbell & Duffey 1979; Barbosa, Gross & Kemper 1991; Thaler 2002; Harvey, van Nouhuys & Biere 2005; Ode 2006; Kazana *et al.* 2007). In situations where herbivores use allelochemicals as an acquired defence against natural enemies and are themselves only negligibly affected by the toxins (Campbell & Duffey 1979; Barbosa *et al.* 1991), the plants may lose the indirect benefits from the action of natural enemies on herbivores. For parasitoids, negative impacts of plant toxins may be either direct (e.g. parasitoids getting in direct contact with toxins accumulating within the herbivorous host tissue) or indirect via changes in e.g. herbivore host size or density. Most of the studies focusing on the mediating effects of plants on herbivore-natural enemy interactions focus on plant secondary compounds that are either permanently expressed or induced by herbivory (Karban & Baldwin 1997). However, plant quality and plant chemistry can also be altered by the presence of microorganisms such as fungal endosymbionts that associate with plants (Clay 1990).

Fungal endosymbionts (= endophytes) are fungi, which complete their whole life cycle within plant tissue (Clay 1990). These endophytes are ubiquitous associates of plant species (Clay 2004; Arnold & Lutzoni 2007). Especially well studied are endophytes of the genus *Neotyphodium* in agronomic grass systems (Saikkonen *et al.* 2006). These endophytes of grasses produce different invertebrate and vertebrate toxic alkaloids (Schardl, Leuchtmann & Spiering 2004). Several studies have shown increased resistance against herbivores of plants harbouring endophytes (e.g. Breen 1994; Bultman & Bell 2003; Hunt & Newman 2005; Meister *et al.* 2006) and associated negative effects on natural enemies (Barker & Addison 1996; Goldson *et al.* 2000; Kunkel & Grewal 2003; de Sassi, Müller & Krauss 2006). However, not all herbivorous insects react equally to the presence of endophytes (Omacini *et al.* 2001; Faeth & Bultman 2002; Saikkonen *et al.* 2006; S. A. Härri, unpublished data) and in some cases there is even clonal variation within one herbivore species in the response to endophytes (A. Bieri, unpublished data). If endophyte-tolerant herbivores could use the mycotoxins as acquired defence against their

natural enemies, they may benefit from the presence of the fungal endosymbionts in the plant resource.

Here, we used an aphid-parasitoid system consisting of an endophyte-tolerant grass aphid *Metopolophium festucae* Theobald and its primary parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae), both of which are commonly found on Perennial ryegrass *Lolium perenne* L. (Krauss *et al.* 2007). Parasitoids are responsible for drastic reductions of aphid densities in the field and thus play a central role in the biological control of pest aphids (Schmidt *et al.* 2003; Brewer & Elliott 2004). *Aphidius ervi* belongs to the subfamily Aphidiinae, which are solitary koinobionts that attack the nymphal stages of aphids and develop within the still growing hosts. Killing of the aphid and mummy formation, in which the parasitoid larvae pupates, normally occurs after the aphids reach adulthood or in a late nymphal stage (Godfray 1994). During the larval growth period, the parasitoid larva feeds on host aphid haemolymph and tissue and may therefore be directly exposed to toxic substances ingested by aphids through the plant sap. Endoparasitoids such as *A. ervi* cannot excrete waste before reaching the final larval instar, as only then the midgut and hindgut fuse. After this fusion, they release the meconium that consists of undigested food and nitrogenous waste (Quicke 1997). As a consequence of this waste product accummulation, the larval stages of parasitoids could be particularly affected by the presence of endophyte-produced toxins. Sex determination of *A. ervi*, as for all hymenopteran parasitoids, occurs through haplodiploidy, with fertilised eggs resulting in females and unfertilised eggs in males. The mated female decides at oviposition whether the egg is fertilised or not and has thus control over the sex of her offspring (Godfray 1994). Theory predicts that unfertilised male eggs are placed into lower quality hosts, whereas fertilised female eggs are laid into higher quality hosts (Charnov *et al.* 1981). As aphids feeding on endophyte infected plants come into contact with the mycotoxins, these host may represent lower quality hosts for the parasitoids and females my decide to place unfertilised, male eggs into such aphids if she can perceive the endosymbiont.

We compared a range of life-history traits of *A. ervi* when offering host aphids feeding either on endophyte-free (E-) or endophyte-infected *L. perenne* (E+). We asked whether the endophyte-tolerant aphid species *M. festucae* can use the mycotoxins that are produced by the plant-endophyte symbiosis as acquired defence against *A. ervi*. Based on the biology of the primary parasitoid *A. ervi*, our main predictions were that the presence of endophytes in the host's food plant 1) reduces the rate of parasitism, 2) results in a sex ratio biased towards males, 3) increases developmental time, 4) increases larval mortality

and 5) reduces longevity of adult parasitoids.

MATERIAL AND METHODS

Material

The seeds of *L. perenne* were provided by Brian Tapper (AgResearch, NZ) and belonged all to the same cultivar (Samson). They were either uninfected (E -; identity number: 11104, < 0.01% infection) or infected with the common wildtype endophyte *Neotyphodium lolii* Glen, Bacon and Hanlin specific to *L. perenne* (E +; identity number: A12038, 89% infection). The infection status of the seed batches was confirmed by a combination of seed staining and microscopic examination, and immunoblotting of stems (see Krauss *et al.* 2007).

The stock culture of *M. festucae* was started in summer 2005 with a few individuals collected near the University of Zürich, Switzerland and was maintained on commercially available endophyte-free fodder grass *L. perenne* ARION (staining of 30 seeds: 0% infection), provided by FAL Reckenholz, Switzerland. The stock culture of *A. ervi* was started with 250 individuals bought from Andermatt Biocontrol AG, Switzerland and maintained on *M. festucae* feeding on *L. perenne* ARION. All stock cultures and experiments were conducted under controlled climatic conditions at 22ºC with a L16 : D8 h light regime.

Experimental set-up

Fifty 1-day old, mated female parasitoids from the stock culture were used for the experiment. For mating, each female was kept in a vial (ø 5 cm x 2 cm height) for 8 hours together with two males of the same age. A piece of apple was added after 4 hours to prevent starvation. After this mating period, each of the 50 females was offered a mixture of 20 second and third instar *M. festucae* nymphs on E- or E+ cuttings in a Petri dish. These nymphs were the progeny of adult *M. festucae* from the stock culture that had been placed on either E- or E+ cuttings in individual Petri dishes. Therefore, these nymphs fed on either E- or E+ plants since their birth.

The 50 mated parasitoid females were left in their Petri dish with the nymphs for a 13 - 15 hour oviposition period overnight. This time span was considered long enough for oviposition to take place but short enough to avoid super-parasitism (S. A. Härri, personal observation). After this oviposition period, the parasitoids were removed and placed in alcohol for later measurements of the dry weight. The dry weight of the *A. ervi* female

mothers in the experiment did not differ between the endophyte treatments ($F_{1,46} = 0.21$, $P = 0.648$) and was therefore not added as an explaining co-factor for statistical analyses. The aphids were removed from the 50 Petri dishes and transferred onto 50 potted plants with either E - or E + *L. perenne* (50 seeds, 6-day-old). The pots were then covered with a cage built from an empty PET - bottle with ventilation slits.

Nine days later, all 50 pots were checked regularly once a day for the presence of mummies. The exact time until mummy formation (= larval development time) in number of days was recorded. The mummies were collected and transferred singly into gelatine capsules. This was repeated for eight consecutive days until all parasitised aphids had mummified. All aphids that did not turn into mummies after 17 days were dissected to assess the cause of parasitoid larval mortality. Dissected aphids were classified as unparasitised or parasitised adults (= surviving adult aphid with a dead parasitoid larvae). The gelatine capsules containing the mummies were checked twice daily for parasitoid emergence. Time from mummy formation to emergence was recorded (= pupal development time) and the total development time was calculated as number of days from oviposition to adult emergence. Emerged parasitoids were sexed and categorized as "healthy" or "crippled". Individuals were assigned to the "crippled" category when they had for example one wing deformed or when they were so weak that they died shortly after emergence within the gelatine capsules. None of the crippled individuals lived long enough to be assessed for longevity (see below). The proportion of healthy parasitoids emerging from mummies was calculated (= emergence rate). The proportion of parasitoids that died during the pupal stage (= pupal mortality rate) is the counterpart of the emergence rate. The sex ratio was calculated as the proportion of male offspring of all offspring per female. Some randomly selected individuals of the emerged females were used for a different experiment. Therefore, for the measurements of longevity and offspring weight, not all emerged "healthy" female parasitoids were tested. On E-, 34 out of 53 emerged female parasitoids and on E+ 29 out of 39 emerged female parasitoids were included in the measurements. All the emerged "healthy" males were tested for their longevity. Longevity was measured by placing each individual singly in a plastic vial closed with a piece of foam. Fresh apple was supplied every day and survival was recorded twice a day. After the death of the parasitoids, the dry weight, after drying them in an oven for 72 hours at 80ºC, was recorded. At the end of the experiment, the size of the aphid mummies was recorded by measuring the length from front of the head to the end of the abdomen, not including the cauda.

Statistical analyses

All analyses were performed with R (version 2.4.0 for Mac OS X). For the endophyte-infected treatment, one replicate was lost at the beginning of the experiment. Three females on E- and three females on E+ did not produce any mummies and these replicates had to be omitted from the analyses. For all analyses, endophyte infection (E-/E+) was included as a fixed effect. The recorded life-history traits were either analysed with one-way ANOVA's (ANOVA), generalised linear model with quasipoisson error structure (GLM, poisson), generalised linear model with quasibinomial error structure (GLM, binomial) or linear mixed effects model including mother identity as a random effect and sex and its interaction with endophyte presence as additional fixed effects (LME). The use of each model is indicated in the Result section. To meet model assumptions of residual normality and heteroscedasticity, pupal survival rate was arcsin-square root-transformed.

RESULTS

The presence of endophytes showed a trend towards lower number of mummies produced by parasitoids on E+ plants and thus possibly fewer aphids attacked (Fig. 1). Dissection of the aphids that did not turn into mummies showed only very few cases where the larvae died within the aphid hosts and there was no significant difference in larval mortality between the two treatments (Fig. 1). The larval development time and the pupal development time were significantly extended in host aphids feeding on endophyte-infected plants, resulting in an increase in total development time on E+ (Fig. 2). Furthermore, female parasitoids took longer to develop than male parasitoids (LME: larval stage: $F_{1,259} = 14.78$, $P < 0.001$, pupal stage: $F_{1,235} = 5.62$, $P = 0.019$, total development time: $F_{1,234} = 17.00$, $P < 0.001$). However, the effect of gender was statistically independent of endophyte infection for larval and pupal development times (LME: larval stage: $F_{1,259} = 0.36$, $P = 0.546$, pupal stage: $F_{1,235} = 0.61$, $P = 0.437$, total development time: $F_{1,234} = 1.32$, $P = 0.252$). The final emergence rate and also the resulting number of emerged and healthy parasitoids were not significantly influenced by the endophyte treatment (Fig. 3). Following from this, the pupal mortality rate was not affected by endophyte presence. The sex ratio of the emerged parasitoids did not differ significantly between the endophyte treatments (E-: 72.62 ± 0.05%, E+: 63.66 ± 0.07%; GLM, binomial: $F_{1,41} = 1.86$, $P = 0.180$). Very few parasitoids were crippled at emergence (Fig. 3). Mummy size was smaller for emerging males than females but did not depend on endophyte infection (Table 1).

The longevity of the emerged parasitoids was neither influenced by the endophyte treatment nor by their gender (Table 1). The weight of the emerged parasitoids did not differ between the endophyte treatments but females were heavier than males independent of endophyte infection (Table 1).

DISCUSSION

The presence of endophytes in the food plant of the aphid hosts increased the larval and pupal developmental time of parasitoids. Unexpectedly, all other life-history traits of the primary parasitoids were not significantly affected by the endophyte environment. Even though the number of mummies tended to be decreased on E+ ($P = 0.078$), the resulting number of emerged offspring was not significantly different between the endophyte treatments. The number of mummies does not stringently reflect the oviposition rate, as parasitoid eggs can get destroyed by the aphids' immune system. Generally, insects can defend themselves against parasitoid attacks by encapsulating the eggs (Kraaijeveld & Godfray 1997). For aphids, the mechanism by which the eggs are destroyed remains unclear but it has been observed that in resistant aphid hosts the eggs do not develop and eventually disappear (Henter & Via 1995; Ferrari *et al.* 2001). As we did not directly observe oviposition and successful placing of eggs can only be detected by dissection shortly after oviposition, we do not know whether attack rates did differ between the treatments.

Comparisons of attack behaviour of different aphid primary parasitoid species show that oviposition and handling time of less than 0.5 seconds for *A. ervi* is very short (Völkl & Mackauer 2000). Short handling times suggest that *A. ervi* does not assess hosts carefully but attacks all available hosts independent of their quality. Therefore, a tendency of a reduced number of mummies formed may reflect differences in the aphids' ability to defend a parasitoid egg before the larva hatches. Other laboratory studies on endophyte effects on parasitoids generally found no differences in attack rates (Barker & Addison 1997; Bultman, McNeill & Goldson 2003; but see Barker & Addison 1996). However, in field experiments, parasitism rate of *Microctonus hyperodae* on endophyte-infected *L. perenne* (Goldson *et al.* 2000) and of *Phyllonorycter* sp. on endophyte-infected *Quercus gambelii* are reduced (Preszler, Gaylord & Boecklen 1996). Under field conditions, where insects have the choice between hosts from endophyte-free or endophyte-infected plants, such differences in attack rate may be caused mainly by preferences of parasitoids for hosts on uninfected plants.

The slow growth - high mortality hypothesis predicts that development time is increased for herbivores feeding on low nutritional plants or plants with allelochemicals and that this extension in development time increases the window of exposure to parasitoids of larval herbivores (Clancy & Price 1987). An extended development time on low quality hosts has also been proposed and shown for parasitoids (Vinson & Iwantsch 1980; Godfray 1994). For example, *M. hyperodae,* a parasitoid of the Argentine stem weevil, has a longer developmental time when developing within hosts feeding on endophyte-infected plants (Barker & Addison 1996; Bultman *et al.* 2003). The larvae of koinobiont parasitoids are intimately associated with and caged in the developing host organism without an option of defence against natural enemies. An extended larval parasitoid development time is thus disadvantageous (Price *et al.* 1980), because it results in an extension of the life stage most vulnerable to attacks by secondary parasitoids and other predatory arthropods (Müller & Godfray 1997; Müller & Godfray 1999; Brodeur & Rosenheim 2000).

The observed increase in larval development time of aphid primary parasitoids on endophyte-infected plants was not observed in another experiment using the same plant-aphid-parasitoid system (S. A. Härri, unpublished data). In that experiment, aphids were kept on live plants during the whole time from oviposition to emergence whereas here, aphids were parasitised on grass cuttings in Petri dishes. The latter may have been stressful for the aphids and it is possible that under stressful conditions aphids are more susceptible to endophyte presence. Additionally, clipping of grasses has been shown to increase the alkaloid concentrations of endophyte-infected grasses (Bultman & Bell 2003). This might explain why we found here but not in the other experiment an increase in developmental time.

Host size and host age are often assumed to be important determinants of the parasitoid's development time (Vinson & Iwantsch 1980; Charnov *et al.* 1981; Sequeira & Mackauer 1992; Godfray 1994). First instars of the aphid species *M. festucae* that served as hosts for *A. ervi* in our experiment do not show any size differences when feeding on endophyte-free or endophyte-infected grasses and their development time is not prolonged in the presence of endophytes (S. A. Härri, unpublished data). Therefore, it is likely that at the time of oviposition, the size of *M. festucae* did not differ between the treatments. At the end of the larval development when mummy formation occurred, the size of the hosts was again not different between the treatments, as indicated by the non-significant differences for mummy size. Therefore, we speculate that host size at

oviposition is not the prime determinant for the observed differences in development time but rather the lower nutritional quality of the host tissue caused by the presence of endophytes. Thus, the observed increase in development time might rather be a direct effect caused by the direct contact with the parasitoid larvae with toxins that possibly accumulated within the host tissue and not an indirect effect via host size differences at time of oviposition or prolonged host development time.

Generally, the results of our experiment show that the effects of endophytes on *A. ervi* are not very strong, because they do not significantly affect oviposition behaviour, rate of parasitism or longevity of emerging parasitoids when their host is feeding on infected plants. However, we found a significant increase in larval and pupal development time for parasitoids from aphid hosts feeding on endophyte-infected *L. perenne*. Therefore, individual aphids are unlikely to acquire effective protection from the endophyte in the food plant, but aphid colonies may achieve benefits in the long term through the extended development time of the parasitoid larvae. Significant extensions of parasitoid development time could ultimately reduce population growth rates of parasitoids and thus lead to lower attack rates of aphid colonies by parasitoids in the presence of endophytes. Additionally, parasitoids developing in endophyte-tolerant aphids may only experience a reduction in reproductive success after developing within the endophyte environment (S. A. Härri, unpublished data).

In conclusion, the endophyte effects proliferate only weakly up the food chain to the aphid parasitoids. In particular, the larva developing within an aphid host feeding on endophyte-infected plants was presumably most exposed to the mycotoxins and as a consequence showed extended development time. Other life history traits, such as longevity and sex ratios showed no effects from the endophyte presence in the basal resource plant. Whether this extension in larval development is caused by the host that uses the toxins to defend a parasitoid larva, by the parasitoid to force its host to acquire the correct size before pupation, or as a side-effect of the accumulated toxins within endophyte-tolerant aphids feeding on infected plants remains to be elucidated by experimentation focusing more precisely on parasitoid larval development.

ACKNOWLEDGMENTS

We would like to thank Julia Nüscheler and Virginie Boreux for the practical help and Swiss Caps AG, Kirchberg, Switzerland for providing the gelatine capsules. The project was funded by a SNSF grant to cbm (Grant number 631-065950).

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TABLES

TABLE 1. Mean ± SE, test statistic, and *P*- values for mummy size, offspring weight and longevity for the parasitoid *A. ervi* developing within the aphid *M. festucae* feeding on endophyte-free (E-) or endophyte-infected (E+) grass *L. perenne*. Test statistics are based on LME with mother identity as random effect. Significant values ($P < 0.05$) are presented in bold.

	E- (mean ± SE)	E+ (mean ± SE)	test-statistic	*P*
Mummy size [μm]	441.15 ± 2.80	440.86 ± 3.14	$F_{1,41} = 0.26$	0.612
sex			$F_{1,253} = 11.48$	**< 0.001**
interaction			$F_{1,253} = 0.97$	0.326
females	446.98 ± 3.72	453.05 ± 3.83		
males	438.67 ± 2.79	437.82 ± 2.95		
Offspring weight [μg]	16.33 ± 0.47	16.75 ± 0.48	$F_{1,40} = 0.02$	0.899
sex			$F_{1,169} = 17.66$	**< 0.001**
interaction			$F_{1,169} = 0.15$	0.702
females	17.81 ± 0.47	18.29 ± 0.54		
males	15.82 ± 0.32	15.66 ± 0.34		
Adult longevity [d]	9.30 ± 0.35	8.61 ± 0.44	$F_{1,40} = 1.24$	0.272
sex			$F_{1,170} = 1.90$	0.170
interaction			$F_{1,170} = 0.44$	0.507
females	9.44 ± 0.54	9.43 ± 0.53		
males	9.08 ± 0.28	8.25 ± 0.30		

FIGURES

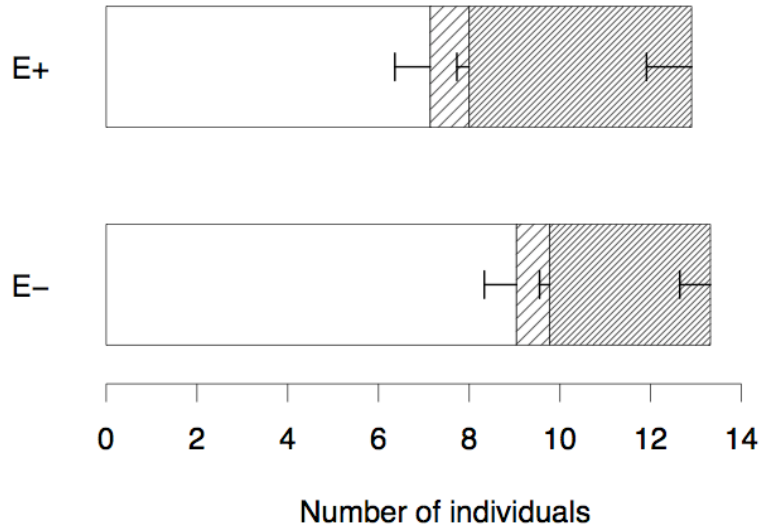

FIGURE 1. Mean (-SE) of number of mummies (white), number of dead parasitoid larvae within aphid hosts (= larval mortality, wide-dashed) and unparasitised aphids (narrow-dashed) on endophyte-free (E-) or endophyte-infected aphid food plants (E+). Number of mummies produced tended to be lower on E+ (ANOVA: $F_{1,41} = 3.27$, $P = 0.078$) whereas larval mortality did not differ between the treatments (GLM, poisson: $F_{1,41} = 0.14$, $P = 0.709$).

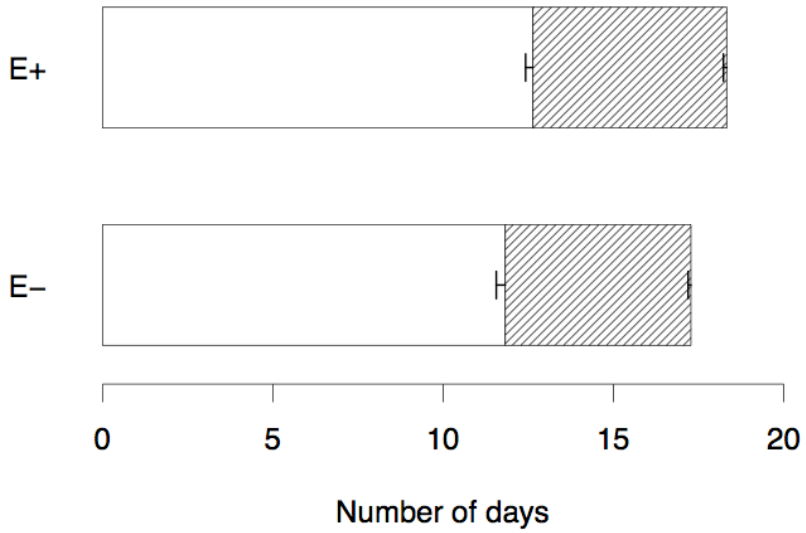

FIGURE 2. Mean (-SE) of parasitoid larval development time (white) and pupal development time within the mummified aphid (dashed) on endophyte-free (E-) and endophyte-infected (E+) plants. Larval (LME: $F_{1,41} = 9.35$, $P = 0.004$) and pupal (LME: $F_{1,40} = 5.02$, P = 0.031) developmental time were prolonged on endophyte infected plants, resulting in an overall increase in total development time on E+ (LME: $F_{1,41} = 10.53$, $P = 0.002$). For additional effects of gender on development times, see text.

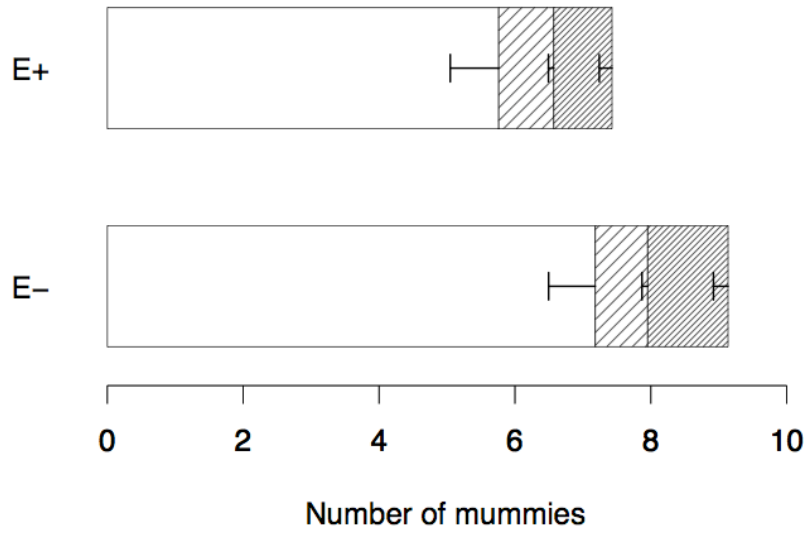

FIGURE 3. Mean (-SE) of number of emerged "healthy" parasitoids (white), number of emerged "crippled" individuals (wide dashed) and number of parasitoid pupae dying (narrow dashed) per female on endophyte-free (E-) or endophyte-infected plants (E+). Number of emerged parasitoids (ANOVA: $F_{1,41} = 2.06$, $P = 0.159$), number of crippled individuals (GLM, poisson: $F_{1,41} = 0.01$, $P = 0.907$) and number of parasitoid pupae dying (ANOVA: $F_{1,41} = 0.65$, $P = 0.426$) were all not significantly affected by endophyte presence

CHAPTER 3

"It is interesting to contemplate an entangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent on each other in so complex a manner, have all been produced by laws acting around us."

CHARLES DARWIN

FUNGAL ENDOSYMBIONTS OF PLANTS REDUCE LIFE SPAN OF HYPERPARASITOIDS AND INFLUENCE HOST SELECTION

SIMONE A. HÄRRI, JOCHEN KRAUSS & CHRISTINE B. MÜLLER

ABSTRACT

Complex biotic interactions within communities shape their structure and dynamics. In studies of multi-trophic interactions, the presence of small, invisible microorganisms associated with plants and the presence of a fourth aboveground trophic level have often been neglected. However, to understand the processes and mechanisms, which act within multi-trophic systems, incorporating these neglected parts is crucial. Here, we ask if and how the presence of a fungal endosymbiont which alters plant quality for aphids and primary parasitoids by producing herbivore-toxic substances trickles up the food chain and affects the performance and host choice behaviour of aphid secondary parasitoids. We offered simultaneously hosts from endophyte-free and endophyte-infected environment to secondary parasitoids. Especially older and more experienced females were able to discriminate against hosts from the endophyte-infected environment. The lower host quality translated into a reduced life span for those secondary parasitoids developing within the hosts from the endophyte-infected environment. Thus, the presence of fungal endosymbionts in the basal resource of a food chain negatively affects the performance of secondary parasitoids. Their discrimination ability might shift the efficiency in limiting primary parasitoids to endophyte- free plants, which co-occur with endophyte- infected plants in natural grasslands.

INTRODUCTION

The mediating effect of plants on the interaction between herbivores and their natural enemies plays a crucial role in structuring natural communities (Ohgushi, Craig & Price 2007). Studies considering these interactions in food chains or food webs tend to focus on only two or three trophic levels (e.g. de Sassi, Müller & Krauss 2006; Tylianakis, Tscharntke & Lewis 2007). However, natural communities often consist of more than three trophic levels (Pimm & Lawton 1977). In insect communities for example, primary parasitoids are commonly attacked by a range of secondary parasitoids (= hyperparasitoids; Sullivan 1987). The presence of hyperparasitoids reduces the limiting

effects of primary parasitoids on herbivores and may disrupt the effectiveness of essential biological control (Rosenheim 1998). Especially in aphid communities, secondary parasitoids are ubiquitous and very diverse and may have a strong impact on interactions among plants, herbivores and primary parasitoids (Müller *et al.* 1999; Sullivan & Völkl 1999). Nevertheless, studies on multi-trophic interactions in insect communities often neglect the presence of the fourth trophic level (Brodeur 2000; but see Harvey, van Dam & Gols 2003; Soler *et al.* 2005).

Apart from consumers at the top of food webs, the presence of microorganisms associating with plants at the bottom of food webs has often been neglected too (Polis & Strong 1996). For example, endophytic fungi (= endophytes) are ubiquitous endosymbionts of a large variety of plant species (Arnold & Lutzoni 2007). Endophytes of the genus *Neotyphodium* are associated with temperate grasses and produce herbivore-toxic alkaloids (Clay 1990). These alkaloids may change food plant quality for herbivores and the effects may cascade up the food chain and propagate to higher trophic levels (Omacini *et al.* 2001; Müller & Krauss 2005; de Sassi *et al.* 2006; S. A. Härri, unpublished data). However, the potential of endophytic fungi to cascade up a food chain and to affect hyperparasitoids has never been investigated experimentally. As hyperparasitoids are intimately linked to primary parasitoids with their adult females (i.e. host feeding) and larvae feeding directly on the pupae of the primary parasitoid (Quicke 1997), it is likely that the presence of endophytes affects the behaviour and performance of hyperparasitoids.

Endophyte infection in natural and agricultural grasslands is common in Europe, with a mosaic of infected and uninfected plants (Saikkonen *et al.* 2000; Zabalgogeazcoa *et al.* 2003). This mosaic of endophyte infection results in spatial heterogeneity of plant quality and such spatial heterogeneity within resources can influence multi-trophic interactions (van Nouhuys & Hanski 2002). If the effects of endophytes propagate to higher consumer levels, the hyperparasitoids may be confronted with a choice of hosts that either feed on aphids from endophyte-free or endophyte-infected grasses. Therefore, if host choice by hyperparasitoids occurs, it may define the impact of the endophyte on hyperparasitoid populations and the structure of the associated aphid-parasitoid community.

Most hyperparasitoids are confronted with a sequence of decisions when encountering a host: should the host be accepted for oviposition, ignored or used as protein rich food? Host choice of parasitoids has been studied theoretically and

experimentally with the majority of the experimental studies focusing on host choice by primary parasitoids whereas theoretical models ignore to distinguish explicitly between primary and secondary parasitoids (Godfray 1994). If eggs were cheap and not limited, and the time required for host attack minimal, parasitoids are predicted to attack all hosts they encounter. However, as parasitoids are either time- and/or egg-limited, oviposition becomes more costly and a precise host choice is important (Heimpel, Rosenheim & Mangel 1996). Most hyperparasitoids are synovigenic, which means that they continue to mature eggs during the adult stage (Quicke 1997). The process of egg maturation requires proteins that are obtained by hostfeeding and slows down with progressing age (Godfray 1994). Therefore, synovigenic parasitoids can get egg-limited either temporarily when deprived of hosts or after encountering a large number of hosts within a short time period or permanently with increasing age (Rosenheim, Heimpel & Mangel 2000). With progressive, age-dependent egg-limitation, females should preferentially oviposit into highest quality hosts (Iwasa, Suzuki & Matsuda 1984) and use lower quality hosts for hostfeeding (Kidd & Jervis 1991). The ability to distinguish between hosts of different quality may depend on the parasitoid's experience (Vet *et al.* 1990). Overall, older and more experienced females are predicted to make more precise host choice decisions than younger ones.

We assumed that primary parasitoids that develop in aphids from endophyte-infected grasses are of reduced quality and predicted that this reduced host quality should influence the host choice behaviour and offspring performance of aphid hyperparasitoids, by (1) increasing larval development time, (2) reducing adult life span, (3) resulting in a male biased sex ratio as theory predicts that fertilized eggs (=females) are laid preferentially into high quality hosts whereas unfertilized eggs (= males) are laid into low quality hosts (Charnov *et al.* 1981), and (4) reducing the weight of adult hyperparasitoids that had developed in such low quality hosts. We further tested whether females are able to distinguish between mummies from endophyte-free (E-) and those from endophyte-infected (E+) environments, depending on age and experience and predicted that (5) older females should be more selective, (6) host experience leads to increased choice of E- mummies for oviposition and (7) the lower quality hosts (E+ mummies) are preferentially used for host feeding.

MATERIAL AND METHODS

Model system

The model system used to address our hypotheses consisted of the endophytic fungi *Neotyphodium lolii* Glen, Bacon, Hanlin, a specialist on the grass *Lolium perenne* L. The aphid species *Metopolophium festucae* Theobald served as hosts for the primary parasitoids *Aphidius ervi* Haliday whose mummies served as hosts for the secondary parasitoids *Asaphes vulgaris* Wlk. The aphid species *M. festucae* is relatively insensitive to the presence of *N. lolii* whereas the fecundity of *A. ervi* is reduced when developing within *M. festucae* feeding on *L. perenne* infected by *N. lolii* (S. A. Härri, unpublished data).

The seeds of *L. perenne* were provided by Brian Tapper, AgResearch, NZ. Seeds were either infected with *N. lolii* (wildtype; E+) or endophyte-free (E-). All of the seeds belonged to the cultivar Grassland Samson. Endophyte infection was lost by selectively choosing plants with unsuccessful endophyte transmission (B. Tapper, personal communication). After the experiment, the endophyte infection status of the plants was verified with the Phytoscreen *Neotyphodium* Immunoblot Assays (Agrinostics Ltd., Watkinsville, USA). On average, 3% of the E- plants were infected whereas 80% of the E+ plants were infected.

The stock culture of *M. festucae* was started in summer 2005 with a few individuals collected close to the University of Zurich, Switzerland. Since then, the culture was kept on *L. perenne* ARION (commercially available endophyte-free fodder grass). The stock culture of *A. ervi* was started with 250 individuals from the company Andermatt Biocontrol AG, Switzerland in Spring 2006. *Aphidius ervi* was maintained on *M. festucae* feeding on *L. perenne* ARION. The stock culture of *A. vulgaris* was started with a few individuals obtained from collected aphid mummies close to the University of Zurich, Switzerland in Summer 2006. The stock culture was maintained on *A. ervi*.

Asaphes vulgaris (Pteromalidae, Chalcidoidea) is an ectoparasitic, solitary mummy parasitoid. Females oviposit on the surface of the pupae of the primary parasitoid and the hatched larva feeds on the primary parasitoid pupa or pre-pupa within the mummified aphid (Christiansen-Weniger 1992). When ovipositing or host feeding, females release venom that kills the primary parasitoid pupae immediately (= idiobionts). The proteins gained from host feeding are allocated to the maturation of eggs. In the absence of hosts, the females can reallocate nutrients from the eggs into the somatic

maintenance by resorption (= oosorption; Godfray 1994). The process of oosorption can lead to egg limitation in synovigenic species (Rosenheim *et al.* 2000).

Experimental set-up

To obtain one-day old mummies from E- and E+ environments, aphids from the stock culture were placed on either E- or E+ pots. After ten days, *A. ervi* females were added and a few days later, the freshly formed mummies were collected over four consecutive days. The explanatory factors were presence of endophytes ('endophyte'), age of *A. vulgaris* females ('age') and *A. vulgaris* female experience ('experience'). The 'endophyte' choice treatment was obtained by offering 14 E- and 14 E+ mummies simultaneously to one female *A. vulgaris* that had no previous experience with E+ or E- environments. The choice experiments were done in Petri dishes and one Petri dish represented one replicate. The 28 mummies were *ad libitum* numbers of hosts, as in all trials some primary parasitoids were emerging from the mummies. The mummies were glued to the Petri dish with a honeywater solution in a checkerboard pattern. To each Petri dish, one female and one male *A. vulgaris* were added. The mating partners had the same age. The factor 'age' was manipulated by using mating pairs of different ages. From the nine mating pairs used in the experiment, the youngest was five days old and the oldest 14 days old with one day difference between each of the pairs (age class 8 was missing). Each of the mating pairs was kept without aphid hosts after emergence until they entered the experimental arena where they were left together with the 14 E- and 14 E+ mummies for 24 hours. The factor 'experience' was obtained by offering each mating pair fresh mummies (14 E- and 14 E+) in a new Petri dish over four consecutive days.

Three days after female and male hyperparasitoids were removed from the Petri dishes the mummies were put individually into gelatine capsules. The gelatine capsules were checked daily for emergence of *A. vulgaris*. The time until emergence (= development time) was recorded. Emerged *A. vulgaris* were sexed and put singly into a plastic vial (5 cm x 2 cm) closed with a foam plug. A fresh piece of apple was provided as sugar source every second day. Time until death (= adult lifespan) was recorded in days. After death, each individual was weighed. The remaining mummies were dissected to detect host feeding or death of the hyperparasitoids larvae. All stock cultures and experiments were conducted under controlled climatic conditions at 22ºC with a L16 : D8 h light regime.

Statistical analyses

All statistical analyses were performed with R (version 2.4.0 for Mac OS X). Means are given as means ± SE throughout the text. To analyse the choice between E- and E+ mummies of each female for successful oviposition (= offspring emerged) and host feeding, the number of emerged *A. vulgaris* and the number of host feeding events from each Petri dish were summed up for each endophyte treatment. This data was analysed using a generalized mixed effects model (glmmPQL – function) with 'experience', 'day', 'endophyte' and all their interactions as fixed effects and 'experience' nested within 'female identity' as random effect using the quasipoisson error structure to account for overdispersion of the count data (Venables & Ripley 2002).

Development time, lifespan and weight of the emerged *A. vulgaris* offspring were analysed with linear mixed effects model (lme – function) with 'age', 'experience', 'sex', 'endophyte' and including all interactions with the exception of 'sex' for which only the interaction with 'endophyte' was included. The factor 'experience' nested within 'female identity' was added as random effect. Differences of the sex of the emerged *A. vulgaris* were analysed using a generalized mixed effects model (glmmPQL – function) including the same fixed and random effects as described above, but excluding 'sex' and its interaction with 'endophyte'.

For all linear mixed effects models the maximum likelihood method was used for model fit and the general positive-definite symmetric variance-covariance structure for the random effects (Pinheiro & Bates 2000).

RESULTS

Test statistics and *P* – values for all measured traits are summarised in Table 1. Seven out of the 9 females reproduced successfully. The choice by *A. vulgaris* female for oviposition was influenced by endophyte presence, with a preference for E- mummies. This resulted in a mean number of offspring of 2.5 ± 0.58 from E- and 1.85 ± 0.34 from E+ mummies. The preference for E- mummies was especially pronounced for the older parasitoids (age x endophyte) and increased with experience (age x experience x endophyte). Overall, the number of mummies used for oviposition increased with the experience of the females (experience) with older females starting to oviposit only after having gained some experience (age x experience, Fig. 1a). Contrary to our expectations, host feeding was not significantly influenced by any of the explanatory factors (Fig. 1b). Mummies from which no primary or hyperparasitoid emerged and that were not used for

host feeding contained dead *A. ervi* larvae that died of unknown causes during development (E-: 4 mummies; E+: 8 mummies).

A total of 40 offspring wasps emerged from E- mummies and 25 from E+ mummies. The development time of *A. vulgaris* larvae from egg at oviposition to adult emergence was not influenced by the presence of endophytes but decreased slightly with progressing age (model estimate ± SE: -032 ± 0.26) and with progressing experience (model estimate ± SE: -0.99 ± 0.45) of their mothers. Further, development time was shorter for males (19.98 ± 0.09 days) than for females (21.33 ± 0.24 days). *Asaphes vulgaris* emerging from E+ had a significantly shorter lifespan than those emerging from E- (Fig. 2). Independent of endophyte infection, females lived significantly longer than males (females: 14.11± 2.73 days, males: 9.37 ± 0.59; Table 1). The only factor that influenced the weight of the emerged adult wasps was their gender (females: 84.67 ± 4.32 μg, males: 65.50 ± 2.27 μg). Contrary to our expectation, the sex ratio of the progeny was not influenced by any of the experimental factors.

DISCUSSION

The adult lifespan of a generalist hyperparasitoid at the top of an aphid-parasitoid food web was decreased when oviposition and larval development took place in hosts experiencing the endophyte environment. Female *A. vulgaris* improved their host choice with progressing age and oviposition experience through selection of more hosts from the endophyte- free environment than younger and less experienced females. Interestingly, offspring performance in endophyte-free hosts was only improved in terms of lifespan and no effects on developmental time, sex or weight were detected. *Asaphes vulgaris* parasitoids are long-lived and synovigenic, maturing their eggs during adulthood. Under such conditions, a significantly reduced lifespan will result in a shorter reproductive time and thus reduced fitness. Similar fitness penalties of endophytes are known for predators and primary parasitoids (Bultman *et al.* 1997; de Sassi *et al.* 2006; S. A. Härri, unpublished data). Ours is the first study showing experimentally that even the top trophic level in this aphid-parasitoid food web can suffer disadvantages from ubiquitous endophytic fungi in grasses. It is also one of very few studies showing that variation in plant quality – here caused by a microbial endophyte – affects individual performance of the fourth trophic level (but see Harvey *et al.* 2003; Soler *et al.* 2005).

In the field where endophyte infection often occurs in a heterogeneous pattern (e.g. Saikkonen *et al.* 2000), a fitness reduction caused by the presence of endophytes

depends on the ability of hyperparasitoids to discriminate against such inferior hosts. We show that more hyperparasitoids offspring emerged from E- mummies than from E+ mummies when females are given a choice. This increase in number of offspring results from an increased number of oviposition events on E- mummies and not from increased larval mortality within E+ mummies as we detected no mortality among hyperparasitoid larvae. Thus, our experiment demonstrates that hyperparasitoids are able to discriminate against hosts from the endophyte-infected environment and this discrimination improves over time and with experience.

The exact cues the females use when selecting the host from the endophyte-free environment must be linked to the aphid mummy as no plants or live aphids were present in the choice arenas. Aphid hyperparasitoids may be attracted to the presence of aphid honeydew (Buitenhuis *et al.* 2004) but aphid honeydew was not present in the choice arenas either. Host acceptance of hyperparasitoids often involves careful, time consuming examination of a mummy by tapping on the mummy and probing the host inside with the ovipositor (Vinson 1976). As mummy size does not differ between E- and E+ (S. A. Härri, unpublished data), the ovipositor that examines the host could receive signals about endophyte-produced chemicals that may have accumulated in the primary parasitoid. Alternatively, the mummified aphid skin could carry clues on past plant associations of the now dead aphid. *Asaphes vulgaris* appears to respond to kairomones that occur in the silky cocoon of aphidiine wasp (Christiansen-Weniger 1992). Independent on the exact cue the female hyperparasitoid receives, host preference has been shown previously for different host species (Chow & Mackauer 1999) and we provide further evidence that age and experience of *A. vulgaris* females plays a decisive role for their reproductive success.

In our experiment discrimination by females against E+ mummies was not a precise behaviour at first with all females ignoring E+ mummies independent of their age and experience. Only the oldest female never oviposited on E+ mummies, while all the others chose hosts from E- but also to a lesser degree from E+ for oviposition (Fig. 1a). The oviposition decision was influenced by the age and the experience of the females with older females attacking more E- mummies than younger females. Overall, with very low experience, almost none of the females oviposited but all concentrated on host feeding for the first couple of days. The oviposition events increased with more experience and increasing numbers of eggs were laid in E- mummies. For host feeding behaviour no such temporal patterns were observed and no evidence for preference was detected.

This lack of detecting hostfeeding patterns may have been caused by the relatively small sample size of nine females and the short time character of the experiment. Experiments with hyperparasitoids are extremely delicate to conduct, which is likely to be one of the main reasons why so little data exists for multi-trophic interactions including hyperparasitoids (Brodeur 2000; but see Harvey *et al.* 2003; Soler *et al.* 2005). We believe that our experiment provides strong evidence for cascading effects of endophytes to the top fourth trophic level despite the small number of females tested. The change in hyperparasitoid number depending on endophyte infection is also supported by a field study (Omacini *et al.* 2001). In the field, all species move freely between endophyte-free and endophyte-infected grass patches. In the study by Omacini *et al.* (2001) the food chain based on endophyte-infected plants and the aphid *Rhopalosiphum padi* showed strong density-mediated effects for primary and secondary parasitoids. The co-occurring aphid *M. festucae* was less affected by the presence of endophytes but its parasitoid community was. In our laboratory experiment, the herbivore aphid *M. festucae* was also relatively insensitive to the presence of the endophytes, while the associated primary (S. A. Härri, unpublished data) and as shown here hyperparasitoids, show reduced fitness in the presence of endophytes. Communities of aphid hyperparasitoids are extremely species rich and future work should include the competitive interactions among these 'top predators' that may alter when energy flows are reduced through the presence of endophytes.

In conclusion, we showed that the fitness of hyperparasitoids when developing within hosts from the endophyte-infected environment was reduced and females were able to discriminate against these hosts of lesser quality. This reduction in fitness of hyperparasitoids in the presence of endophytes limits their top-down control on primary parasitoids and may result in complex indirect effects for lower trophic levels in the food web.

ACKNOWLEDGMENTS

We thank Dennis Hansen for helpful discussions and comments on the manuscript. The project was funded by a SNSF grant to cbm (631-065950).

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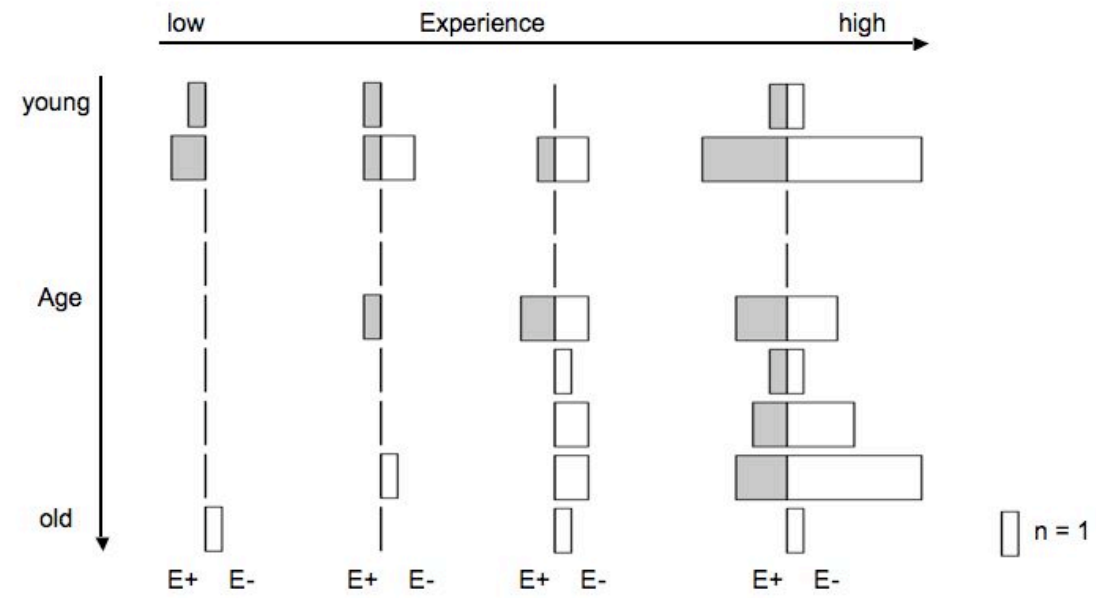
TABLE 1. The influence of age, experience, sex, endophyte and the interactions of interest on the life-history parameters of *A. vulgaris*. The last two columns, oviposition and host feeding, refer to the adult female generation that had a choice between mummies from E+ and E- environments. Here, the explanatory factors age, experience, endophyte and the interactions were tested. The significant results are presented in bold letters.

	Development	Life span	Weight	Sex	Oviposition	Host feeding
Age	$\mathbf{F_{1,5} = 7.00}$ $\mathbf{P = 0.046}$	$F_{1,5} = 0.19$ $P = 0.682$	$F_{1,5} = 2.72$ $P = 0.160$	$F_{1,5} = 0.60$ $P = 0.475$	$F_{1,7} = 0.55$ $P = 0.482$	$F_{1,7} < 0.001$ $P = 0.997$
Experience	$\mathbf{F_{1,10} = 6.97}$ $\mathbf{P = 0.025}$	$F_{1,10} = 3.37$ $P = 0.096$	$F_{1,10} = 0.69$ $P = 0.425$	$F_{1,10} = 0.53$ $P = 0.484$	$\mathbf{F_{1,25} = 37.8}$ $\mathbf{P < 0.001}$	$F_{1,25} = 0.16$ $P = 0.696$
Sex	$\mathbf{F_{1,36} = 35.92}$ $\mathbf{P < 0.001}$	$\mathbf{F_{1,36} = 12.32}$ $\mathbf{P = 0.001}$	$\mathbf{F_{1,36} = 9.46}$ $\mathbf{P = 0.004}$	-	-	-
Endophyte	$F_{1,36} = 0.97$ $P = 0.331$	$\mathbf{F_{1,36} = 7.22}$ $\mathbf{P = 0.011}$	$F_{1,36} = 0.27$ $P = 0.606$	$F_{1,40} = 0.47$ $P = 0.499$	$\mathbf{F_{1,32} = 4.81}$ $\mathbf{P = 0.036}$	$F_{1,32} = 0.13$ $P = 0.719$
Age x Experience	$F_{1,10} = 0.50$ $P = 0.496$	$F_{1,10} = 1.81$ $P = 0.208$	$F_{1,10} = 0.01$ $P = 0.917$	$F_{1,10} = 1.03$ $P = 0.335$	$\mathbf{F_{1,25} = 6.82}$ $\mathbf{P = 0.015}$	$F_{1,25} = 1.62$ $P = 0.214$
Age x Endophyte	$F_{1,36} = 0.01$ $P = 0.909$	$F_{1,36} = 2.59$ $P = 0.116$	$F_{1,36} = 0.43$ $P = 0.515$	$F_{1,40} = 1.64$ $P = 0.208$	$\mathbf{F_{1,32} = 6.29}$ $\mathbf{P = 0.017}$	$F_{1,32} = 1.56$ $P = 0.221$
Experience x Endophyte	$F_{1,36} = 0.17$ $P = 0.683$	$F_{1,36} < 0.01$ $P = 0.971$	$F_{1,36} = 0.07$ $P = 0.792$	-	$F_{1,32} = 1.75$ $P = 0.195$	$F_{1,32} = 1.00$ $P = 0.325$
Sex x Endophyte	$F_{1,36} = 2.68$ $P = 0.111$	$F_{1,36} = 2.54$ $P = 0.120$	$F_{1,36} = 0.43$ $P = 0.515$	-	-	-
Age x Experience x Endophyte	$F_{1,36} = 2.48$ $P = 0.124$	$F_{1,36} = 0.07$ $P = 0.797$	$F_{1,36} = 2.87$ $P = 0.099$	-	$\mathbf{F_{1,32} = 8.42}$ $\mathbf{P = 0.007}$	$F_{1,32} = 1.62$ $P = 0.212$

FIGURES

a)



b)

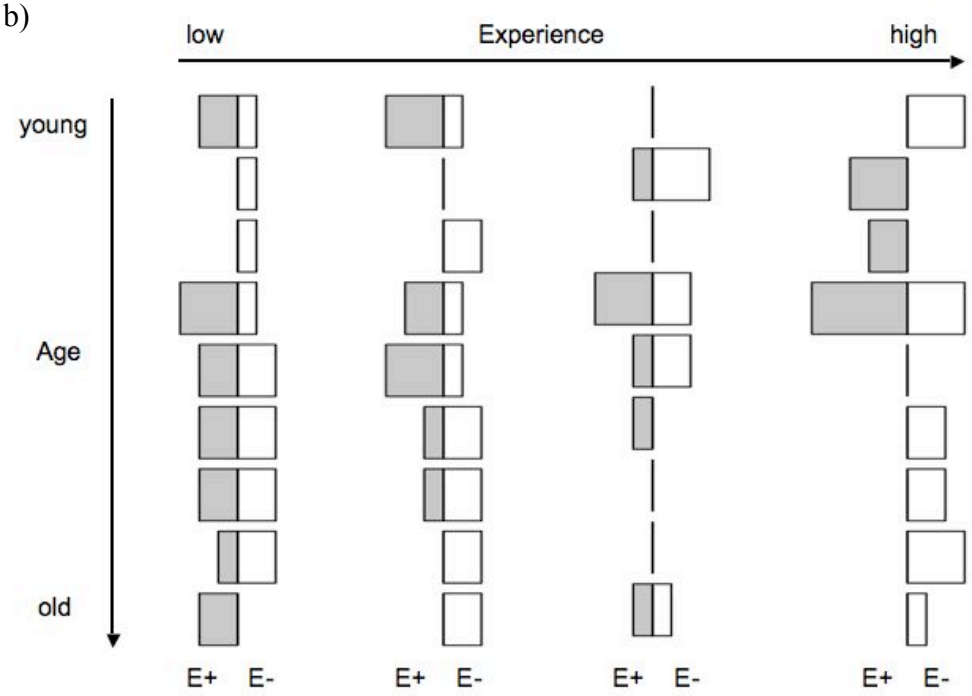


FIGURE 1. The presence and the quantity of a) oviposition events and b) host feeding events on E- (white bars) and E+ (grey bars). From left to right are the four days ('experience') and from top to down are the different ages of the females (excluding age class 8). The data shows the increase in oviposition probability over the four days and an increase of oviposition probability with increasing age (see also Table 1). Two females (aged 7 and 9 days at the beginning of the experiment) were not ovipositing despite showing host feeding behaviour.

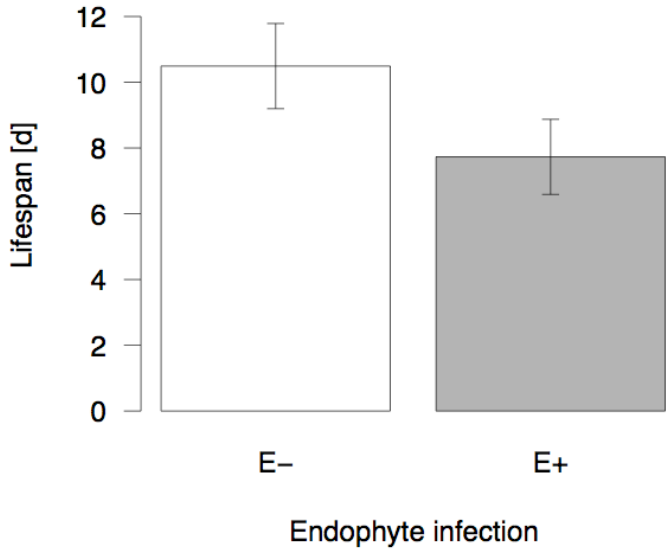


FIGURE 2. The mean ± SE lifespan for *A. vulgaris* offspring emerging from E- mummies (white bar) and E+ mummies (grey bar). The mean was calculated from the average of each Petri dish. The statistics are presented in Table 1 (column 'Lifespan').

CHAPTER 4

"Zwei Seelen wohnen, ach, in meiner Brust"

JOHANN WOLFGANG VON GOETHE

ENDOPHYTIC FUNGI DECREASE AVAILABLE RESOURCES FOR THE APHID *RHOPALOSIPHUM PADI* AND IMPAIR THEIR ABILITY TO INDUCE DEFENCES AGAINST PREDATORS

TOBIAS ZÜST, SIMONE A. HÄRRI & CHRISTINE B. MÜLLER

ABSTRACT

1. The production of winged morphs is a well known mechanism of induced defence in aphids to escape from natural enemies, and is also a reaction to poor resource quality.
2. Host plants of aphids often associate with endophytic fungi that have been shown to reduce the fitness of some species of aphids.
3. We hypothesised that endophyte infection of host plants that represents a low quality plant resource should increase the aphid's induced response to a predator because both, low plant quality and predator presence represent a stronger cue for wing production than predator presence alone.
4. In a laboratory experiment bird cherry-oat aphids *Rhopalosiphum padi* L. were exposed to the factors 'predator threat' and 'endophyte infection' and the effects of these factors on the proportion of winged morphs produced by the aphid colonies was analysed.
5. The presence of endophytic fungi strongly decreased aphid colony sizes. When a predator threat was present all colonies on endophyte-free grasses produced winged morphs whereas only a few colonies were able to produce winged morphs on endophyte-infected grasses. However, these few colonies produced larger proportions of winged morphs than colonies on endophyte-free grasses. Without a predator threat, no colonies on endophyte-infected grasses produced any winged morphs.
6. These results show that aphids in stressed conditions and with reduced fitness will only invest in inducible defences when predators are present but are unable to produce winged morphs in response to endophyte presence.

INTRODUCTION

Animals and plants have evolved various traits and mechanisms to reduce risks of predation and herbivory. Inducible defences are a class of defence mechanisms that are only expressed when a threat by a predator or a herbivore is eminent. This contrasts to constitutive defences that are expressed permanently. Therefore, inducible defences have

the benefit of maximising fitness because the investment in defence traits only occurs when they are needed. Inducible defence responses include several behavioural and morphological traits that increase the victim's resistance against predator attack, reduce predator encounter probabilities and increase the escape probability after predator attack (Tollrian & Harvell 1999). For example, various zooplankton species such as water fleas of the genus *Daphnia* produce defensive structures, such as spines and helmets, to increase their resistance against predator attack. These defensive structures are only expressed when *Daphnia* are exposed to predators, because building the structures reduces their longevity (Harvell 1992; Tollrian 1995). In tadpoles of various amphibian species, inducible defences are present as behavioural or morphological changes. Tadpoles will reduce their foraging time in the presence of a predator to decrease predator encounter probability. As a second response, the tadpoles will grow a larger tail fin, which will mislead predators to attack the tail rather than the vital forepart of the tadpole, thus increasing the tadpole's escape probability. Both induced defence mechanisms reduce resources available for tadpole growth and will delay metamorphosis (van Buskirk & McCollum 2000).

Life-history theory predicts a trade-off between optimal predator defence and the victim's fitness (Stearns 1992; Steiner & Pfeiffer 2007). If the defence would not have a cost it would be permanently expressed, thus being constitutive (Tollrian & Harvell 1999). The intensity of the trade-off between inducible defence and fitness depends on the probability of predator encounters in a given environment and the actual costs of building the defence mechanism. If initiated quickly, inducible defences are superior to constitutive defences in environments with unpredictable predator attacks that, once initiated, are sustained long enough for the defence to become effective (Clark & Harvell 1992; Riessen 1992; Adler & Karban 1994). Inducible defences are often triggered by substances secreted by predators called kairomones (Tollrian & Harvell 1999). Alternatively, the triggering factors of inducible defences can be pheromones that are secreted as alarm signals by prey individuals sensing an imminent threat or by victims of a predator or parasite attack (Nault, Edwards & Styer 1973; Kunert *et al.* 2005).

Aphids (Homoptera: Aphididae) are cyclical parthenogenetic with asexual reproduction during most of the year and sexual reproduction in autumn, thus a colony of aphids consists of mostly clonal individuals with identical genomes (Lushai *et al.* 1997). Deteriorating nutritional conditions, crowding and changes in photoperiod and temperature all result in a higher proportion of winged morphs within an aphid colony

(Sutherland 1967; Dixon & Wratten 1971; de Barro 1992; Müller, Williams & Hardie 2001). This is possible because aphids can produce individuals with different morphologies asexually (Dixon 1998). The production of winged morphs is important for aphid colonies because it enables a clone to disperse and find new resource plants when, for example, food resources deteriorate. The production of winged morphs is also a reaction to the presence of natural enemies and thus a form of induced defence (Dixon & Agarwala 1999; Weisser, Braendle & Minoretti 1999).

Wing production as an inducible defence is triggered by alarm pheromones that most aphids secrete from their siphunculi when attacked by enemies (Mondor & Roitberg 2004; Kunert *et al.* 2005).These pheromones can be perceived by other aphids as far as three centimetres away (Nault *et al.* 1973). Releasing alarm pheromones within a clonal colony of aphids is likely to increase the inclusive fitness of the signaller as the cue will reach closely related individuals. It has been demonstrated that aphids preferably emit alarm pheromones when surrounded by aphids of the same clone as opposed to aphids of other species (Robertson *et al.* 1995). There is a cost for growing wings, because although winged morphs have a higher chance of escaping bad conditions or a high predator risk environment, they are less fecund (Dixon & Wratten 1971) and develop slower than wingless morphs (Dixon 1998). These trade-offs explain why most aphid species do not express the winged morph type constantly.

Most plant species that accommodate aphids have evolved alliances with microorganisms that can alter the plant's quality (Arnold *et al.* 2000; Clay 2004). In particular, the association with endophytic fungi of the genus *Neotyphodium* can lead to the production of alkaloids by the fungus which renders the grass toxic to herbivores (Clay 1988; White, Morgan-Jones & Morrow 1993; Breen 1994; Müller & Krauss 2005). The effects of such mycotoxins also move up the food chain and reduce the fecundity of predators and parasitoids (de Sassi, Müller & Krauss 2006; Härri, S.A., unpublished data). We are not aware of any studies that investigated the effects of endophyte presence and occurrence of mycotoxins in the plants on the induction of winged aphid morphs although such a response to the low quality of infected plants is conceivable if aphids perform worse on infected than on uninfected plants.

In our experiment we addressed whether wing induction as an inducible defence in aphids against predators is altered by the presence of endophytes in the plant. We studied the bird cherry-oat aphid *Rhopalosiphum padi* L., for which endophyte presence reduces lifespan and fecundity, and thus fitness (Meister *et al.* 2006). We hypothesised that wing

induction is *(1)* generally increased on endophyte-infected plants because such plants are of lower nutritional quality than uninfected plants and *(2)* that the inducible defence expressed as increased wing production under high predation risk is also increased for aphids on infected plants because they experience both, toxic food and predator presence. We used a crossed factorial design with endophytes and predators either present or absent to test for possible interactions. We predicted highest proportions of winged morphs when both a predator and the endophyte are present.

MATERIALS AND METHODS

Plants, aphids and ladybirds

The experiment was carried out on tall fescue (*Lolium arundinacea* Schreb.; cultivar Kentucky 31), kindly provided by Prof. Keith Clay (Indiana University, Bloomington, USA). Half of the seeds were uninfected (E-) and half were infected (E+) with the endophytic fungi *Neotyphodium coenophialum.* The infection status was confirmed after termination of the experiment as explained below.

The seeds were planted in plastic seed trays three months before the start of the experiment. At the start of the experiment the plants were cut to a length of 15 cm and 120 (60 E+ and 60 E-) randomly chosen single plants were replanted in plastic pots (Ø10 cm) filled with commercially available gardening compost. Each pot contained three single plants, resulting in 20 pots with infected and 20 pots with uninfected *L. arundinacea* Kentucky 31.

Rhopalosiphum padi individuals were taken from a base culture that had been initiated from three clones. The base culture had been maintained in a climatic chamber for over five months on endophyte-free perennial ryegrass *Lolium perenne* L. (commercially available cultivar ARION), thus the culture was most likely dominated by one clone only.

Seven days after replanting, 15 adult *R. padi* from the laboratory culture were transferred onto each of the 40 pots. The pots were then covered with air-permeable cellophane bags (16 cm x 30 cm) that were attached to the rim of the pots with adhesive tape.

The larvae of the two-spot ladybird *Adalia bipunctata* L. that represented the predation threat were bought from a commercial supplier (Biocontrol Andermatt AG, Grossdietwil, Switzerland).

Experimental set-up

For the experiment, the ladybird larvae were kept in small gauze bags (5 cm x 10 cm) together with aphids serving as food. Thus the experimental aphid colonies received all cues of a foraging predator nearby without decreasing their numbers by direct predation. Twenty bags were prepared, each containing one larvae of *A. bipunctata* and approximately 50 individuals of *R. padi* on cut grass blades (ARION). The gauze bags were sealed with pieces of thin wire and placed within half of the E- pots and half the E+ pots (P+). The other half of the pots (10 E+ and 10 E-) served as controls with empty gauze bags inside the cellophane bags (P-). Every second day, the ladybird larvae were provided with 50 new prey aphids by opening the cellophane bags and removing the gauze bags. The gauze bags were opened, the dry grass and aphid carcasses removed and bags were restocked with new aphids on fresh grass before placing them back on the pots. As this procedure may have caused some disturbance to the experimental aphids, the same procedure was done to the control groups (P-).

The experiment was carried out in a controlled environment chamber (22° C and 16:8 light:dark cycle) with pots randomly arranged. The set of pots with predators (P+) were placed approximately one metre away from the control plots (P-). Pheromones of aphids are only transmitted over short distances (Nault *et al.* 1973), so the control plots (P-) could not have been affected.

After aphids in the experiment were exposed to the predators for 10 days, the first larvae of *A. bipunctata* reached their pupal stage and all the gauze bags were removed. After another day the grass was cut just above soil level and put in the cellophane bag that covered the pot previously to ensure minimal losses of aphids. The cellophane bags were sealed and frozen for later counting of the aphids. The number of *R. padi* individuals was recorded for all replicates. The developmental stage of the aphids was determined under a binocular microscope. The first to third instars were grouped as nymphs, because winged morphs cannot be determined until the aphids reach the fourth instar. Winged fourth instar *R. padi* can be differentiated easily from wingless fourth instars by the presence of wing buds. All fourth instars with wing buds and adults with wings were grouped as winged and all remaining fourth instars and wingless adults were grouped as wingless morphs.

After the experiment, all 120 grass plants of the 40 pots were analysed with Phytoscreen *Neotyphodium* Immunoblot Assays (Agrinostics Ltd., Watkinsville, USA) to confirm endophyte infection. From the parts that had been left when the grasses were cut to remove the aphids, a cross section of the base tiller was extracted with a razor blade.

After carrying out the assay, the immunoblot card was photographed with a Canon EOS 350D digital camera and the tiller tissue imprints were analysed for colour intensity as described by Koh *et al.* (2006). The measured intensity was compared with the reference sample of *Neotyphodium* provided on the immunoblot card. Assuming for the reference sample an infection of 100 %, this procedure allowed us to exclude all replicates with one or more grass tillers of an infection above 20 % for E- and all replicates with one or more grass tillers with an infection of less than 20 % for E+. On E-, six pots (three on P- and three on P+) and on E+, three pots (two on P- and one on P+) had to be omitted from the analysis because their infection status was inadequate.

Statistical analyses

Statistical analyses were performed in R (version 2.3.1 for Windows XP). Data of absolute number were tested for normality of the residuals and equality of variances and had to be ln[x+1]-transformed. The number of aphids per replicate was tested by a two-way ANOVA with 'endophyte infection' and 'predator threat' as fixed effects.

For the winged morphs we tested first the influence of the explanatory variables on the occurrence (presence or absence) of winged morphs and secondly the influence on the proportion of winged morphs for the replicates with at least one winged morph present. This separation was necessary because including all replicates into the analysis of proportion of winged morphs violated the model assumption of variance homogeneity caused by the result that none of the replicates in the E+P- treatment produced any winged morphs. The proportion of winged morphs was analysed instead of absolute numbers to correct for aphid population size. Proportions of winged morphs were calculated by dividing the number of winged morphs (forth instar and adult stage) by the sum of all forth instar and adult stages. Nymphs were not included in this calculation as they may turn into either of the two morphs. In our analyses we included colony size as a co-variable to distinguish between the strong reduction in colony size caused by the endophyte and the independent, direct effect of endophytes on the occurrence and proportion of winged morphs. The interactions with colony size were not included, as model comparison tests showed no improvement of the model fit. The occurrence of winged morphs was analysed using a generalized linear model (GLM) with ‘colony size’, 'endophyte infection', 'predator threat' and the interaction between ‘endophyte infection’ and ‘predator threat’ as factors using a quasibinomial error structure to account for overdispersion (Crawley 2002). The proportion of winged morphs for the replicates

producing at least one winged morph was analysed by the same generalized model as described above. For the non-significant interaction term in the model of winged morph occurrence, we performed a Fisher's exact test.

RESULTS

The final size of the aphid colonies was affected by both, endophytes and predators. Overall, aphid colonies performed poorly on infected grasses. Within endophyte infection groups, aphid colonies exposed to predators reached larger colony size (Fig. 1) and produced higher proportions of winged morphs (Fig. 2) than those without predator threat. Both endophyte infection and predator threat had strong significant effects on aphid colony size and there was a significant interaction between the two factors (Table 1).

Rhopalosiphum padi produced winged morphs in most replicates on E- (P-: 6 out of 7; P+: 7 out of 7), but on E+ winged morphs were observed in a few replicates only (P-: 0 of 8; P+: 3 of 9). This decreased probability of occurrence of winged morphs on E+ was partly caused by the smaller colony sizes but also by endophyte infection independent of the colony size (Table 1). The effect of the endophyte presence on the reduced probability of the production of winged morph was independent of the presence of a predator threat (Table 1). The non-significant interaction term was confirmed by the Fisher's exact test on the independence of number of replicates with winged morphs present between endophyte infection and presence of a predator threat ($P = 0.25$). Also, the presence of a predator threat did not increase the probability that a colony produced winged morphs (Table 1).

Considering only colonies that produced at least one winged morph, the proportion of winged morphs per total number of adult and fourth instar aphids were significantly different in the four treatments with much higher proportions of winged morphs in the P+ treatments (Fig. 2; Table 1). Larger colonies contained disproportionately higher proportions of winged morphs, but endophyte infection also led to a slight increase in the proportion of winged morphs if a predator threat was present (Table 1). The interaction between 'endophyte infection' and 'predator threat' could not be calculated because none of the replicates on E+ without predator threat (P-) did produce winged morphs.

DISCUSSION

Both, the presence of endophytes and that of a predator threat influenced the production of winged morphs. Predator threat mainly increased the proportion of winged morphs within colonies that were able to produce winged morphs whereas endophyte infection reduced a colony's ability to produce any winged morphs. This reduction in the colony's ability to produce winged morphs was mainly but not only caused by the fact that the colonies on infected plants stayed relatively small and performed poorly independently of the presence of a predator threat. The endophyte infection also had effects on wing induction independent of the reduced colony size, possibly because infected grasses represent inferior resource quality. These results contradict our initial hypothesis that cues for wing induction may be increased on infected plants with an additional predator threat, because although the proportion of winged morphs on E+P+ was slightly higher than on E-P+, only few colonies on E+P+ did produce winged morphs at all.

On endophyte-free grass, *R. padi* produced a low proportion of winged morphs of about five percent when no predator was present in six of seven colonies. This proportion might be a response to crowding but it is also possible that *R. padi* always produces small proportions of winged morphs as a form of prudent behaviour. Such low levels of winged morph production may prevent that a predator destroys a colony completely as there are always winged dispersers that can quickly initiate a colony elsewhere when a predator attack is imminent.

When *R. padi* fed on infected grasses without a predator threat, none of the colonies produced winged morphs. However, with a predator present one third of all colonies produced winged morphs with proportions slightly higher than those of the colonies on uninfected grasses. A possible explanation for this may be that most of the aphids on infected grass chose not to reproduce. Meister *et al.* (2006) showed that feeding on endophyte-infected grasses reduces lifespan and fecundity of *R. padi*, thus endophyte-infected grasses represent very poor-quality hosts for this species of aphid. In the field, these aphids may walk away from infected plants as they are able to walk as far as 180 cm to colonize new plants (Alyokhin & Sewell 2003). Nevertheless, on-soil dispersal is risky as the aphids are exposed to a wide range of epigeic predators (Griffiths, Wratten & Vickerman 1985; Sunderland, Fraser & Dixon 1986) and even one winged disperser may increase their chances of colonising new resources considerably. This might explain why the proportion of winged morphs was highest on E+P+. Aphids that feed on endophyte-infected grass and are threatened by a predator should leave their host plant immediately.

In contrast, aphids on endophyte-free grass of adequate resource quality will also invest part of their resources in wingless morphs to ensure better survival of the local clone.

Our results suggest that the strong negative effects on *R. padi* colony size by endophytes in another field study (Omacini *et al.* 2001) were unlikely to be caused by increased production of winged dispersers, as the increase in proportion of winged morphs on E+P+ was very small in absolute numbers (Fig. 1). On the contrary, the low aphid densities on infected plants in the field are most likely caused by reduced survival on such plants or by emigration of wingless morphs.

A possible caveat of our experiment was that we did not control for aphid presence in bags in the control treatment (P-) but placed empty bags only. It is conceivable that aphids in bags might have produced some signals when dying that could have affected the wing induction of our target colonies. Furthermore, we did not control for clonal identity of the experimental aphids although there could be clonal variation in wing morph production. However, as the aphids were randomly distributed over the treatments, possible differences in clonal variation would increase the overall variance and thus not distort the observed pattern.

We showed that *R. padi* can increase winged morph production in the presence of predators as has been demonstrated for the pea aphid, *Acyrthosiphon pisum* (Dixon & Agarwala 1999; Weisser *et al.* 1999; Sloggett & Weisser 2002; Kunert & Weisser 2003). Little is known about the underlying molecular mechanisms that lead to wing induction in aphids but it is likely that growing wings or producing winged offspring after reception of alarm pheromones is under neural control and represents a ‘decision’ of individual aphids (Dixon 1998). If the low proportion of winged morphs on E-P- in our experiment is indeed a result of prudent behaviour, then the lack of any winged morphs on E+P- and their presence on E+P+ would show this decision-making ability of *R. padi* as they would have to be able to assess threat level and nutritious condition.

Predator threat in our experimental colonies not only increased the proportion of winged morphs but also the total number of aphids compared to colonies without predators. This increase in colony size could be a result of increased reproduction as a response to the predator threat, a mechanism of reproductive compensation that has been demonstrated for snails exposed to trematod parasites (Minchella 1985). Parasitized snails increase their reproduction immediately following parasite exposure. It is possible that reproductive compensation exists in aphids as well as increasing reproduction might be a good strategy to compensate for predator attacks if predators do not kill all aphids on a

plant. Adult ladybirds generally leave a plant before all aphids are eaten (Minoretti & Weisser 2000) and ladybird larvae reach their pupal stage after some time during which they do not consume any more aphids. Depending on the magnitude of the response, fecundity compensation might countervail the losses caused by a foraging predator. However, reproductive compensation must have a cost as otherwise all aphids should reproduce at a higher rate, even those that are not exposed to a predator threat. The cost may be smaller birth weight/size of the nymphs, but unfortunately our experimental design did not allow us to measure birth weight of nymphs. Reproductive compensation when exposed to a predator could explain our results of larger colony size in P+ treatments. We could detect this effect only because we used non-lethal predators, i.e. predators that did not feed on target colonies. Experiments that calculated aphids eaten by predators during the experiment might have underestimated these numbers when neglecting fecundity compensation (Weisser *et al.* 1999; Kunert & Weisser 2003).

The expression of inducible defences depends on fitness costs and available resources. We showed that on low quality resources the inducible defence might not be expressed unless a predator threat is present and defence is immediately needed. We also suggest that besides wing morph production as a response to predator threats there may be reproductive compensation by aphids in response to predator presence. When available resources are too restrictive and predators absent, aphid colonies perform very poorly and are unable to induce increased wing morph production. Inducible defences may be superior to constitutive defences as they represent a way for an individual to invest in different defence strategies as required. They thus increase survival in harsh environmental conditions and may be a reason for their evolutionary success in many different organisms.

ACKNOWLEDGMENTS

Wolfgang Nentwig, Tom Jones and two anonymous reviewers provided helpful comments and improvements on the manuscript and Jochen Krauss helped planning the experiment. Further thanks go to Bernhard Schmid for statistical assistance and Keith Clay for providing seeds of Kentucky 31. The study was funded by the Institute of Environmental Sciences, University of Zürich and a SNF grant to CBM (No 631-065950).

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TABLES

TABLE 1. Results of the generalized linear models showing the effects of colony size, endophyte infection ("Infection") and predator presence ("Threat") on the occurrence of winged morphs (colonies producing winged morphs yes/no) and on the proportion of winged morphs within all colonies that produced winged morphs. From this proportion, the interaction could not be calculated, because on endophyte-infected plants without predators no winged morphs were produced at all. The total number of aphids (total colony size; ln[x+1] - transformed) was analysed with a two-way ANOVA with "Infection" and "Threat" as explanatory variables.

	Colonies producing winged morphs yes/no	Proportion of winged morphs	Total colony size
Colony size	$F_{1,26} = 123.32$ $P < 0.0001$	$F_{1,12} = 7.23$ $P = 0.020$	-
Infection	$F_{1,26} = 8.95$ $P = 0.006$	$F_{1,12} = 6.22$ $P = 0.028$	$F_{1,27} = 81.08$ $P < 0.0001$
Threat	$F_{1,26} = 0.45$ $P = 0.507$	$F_{1,12} = 15.58$ $P = 0.002$	$F_{1,27} = 23.92$ $P < 0.0001$
Infection x Threat	$F_{1,26} = 0.00$ $P = 1.00$	NA	$F_{1,27} = 4.92$ $P = 0.035$

FIGURES

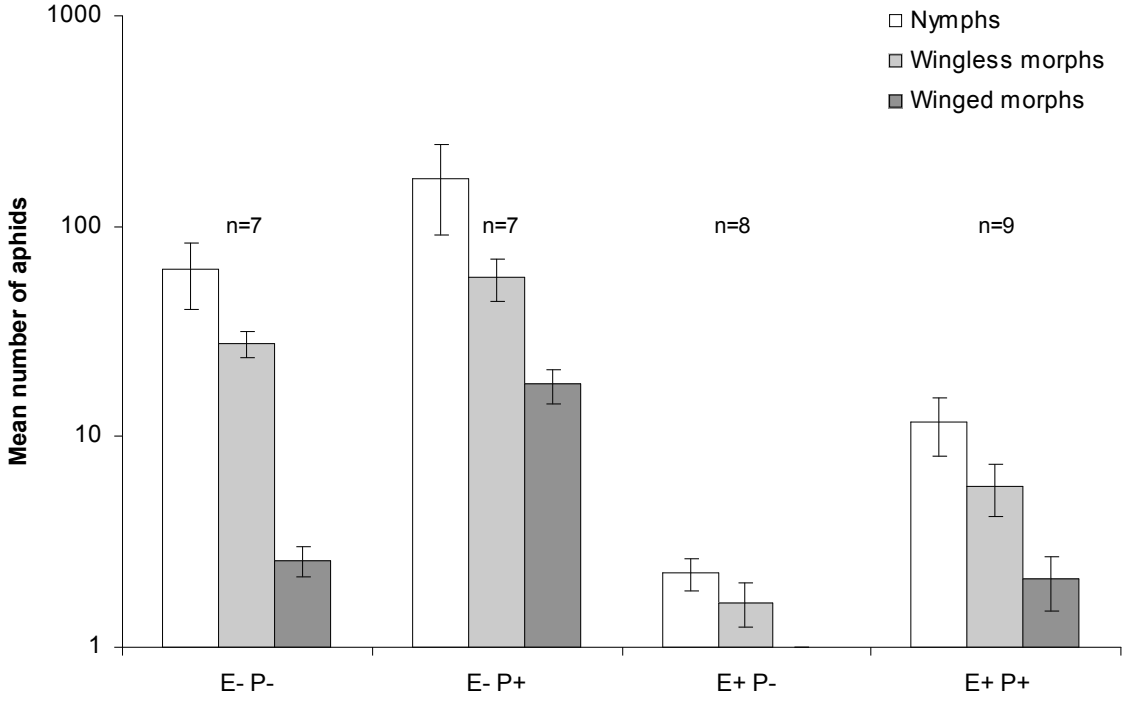

FIGURE 1. Mean (± SE) number of aphids on endophyte-free (E-) and endophyte-infected (E+) *L. arundinacea* with either a predator present (P+) or absent (P-). Note the logarithmic scaled y-axis. The numbers of aphids are categorized into number of nymphs (white bars), number of wingless (grey bars) and winged (dark grey bars) morphs ("n" indicates of the number of replicates after omitting pots with the wrong infection status).

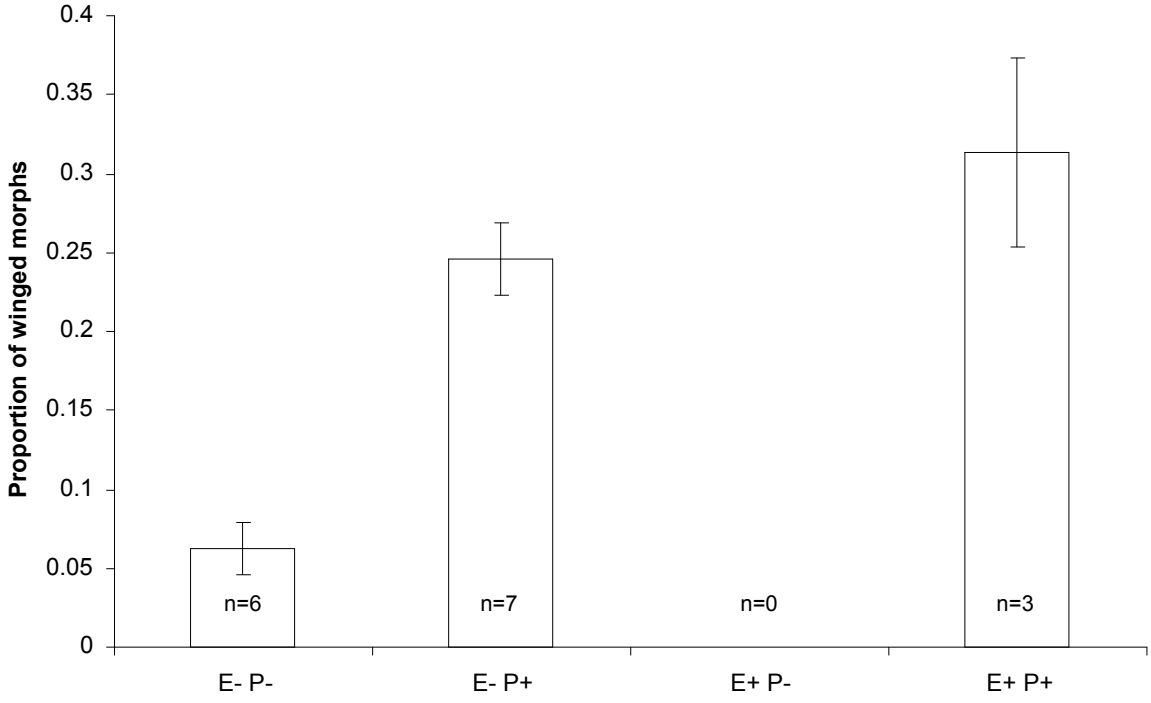

FIGURE 2. Mean (± SE) proportion of winged aphids on endophyte-free (E-) and endophyte-infected (E+) *L. arundinacea,* either in the presence of a predator (P+) or without a predator (P-). The proportions were only calculated for colonies that produced at least one winged individual ("n" indicates the number of replicates with winged morphs present).

CHAPTER 5

"There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved."

CHARLES DARWIN

BETWEEN-CLONE VARIATION IN APHID PERFORMANCE WHEN EXPOSED TO RESOURCES ASSOCIATED WITH FUNGAL ENDOSYMBIONTS

ATLANT P. S. BIERI, SIMONE A. HÄRRI, CHRISTOPH VORBURGER & CHRISTINE B. MÜLLER

ABSTRACT

In asexual species, clonal variation within populations may be maintained by fitness differences that depend on environmental conditions. For herbivores, such as aphids that reproduce mostly asexually, plant quality and variation in chemical plant defences may maintain clonal variation in performance. Apart from plant secondary compounds, liaisons between plants and symbiotic fungi can produce herbivore-toxic compounds that represent acquired chemical protection of the plants. We demonstrate genetic variation among aphid genotypes in response to such fungi by comparing life-history traits of 37 *Rhopalosiphum padi* clones feeding on endophyte-free or endophyte-infected *Lolium arundinaceum*. Most of these clones performed better in the endophyte-free environment, but there were a few clones that performed better in the endophyte-infected environment. We found no trade-offs between the performance on infected and uninfected plants, which indicates superiority of some clones in both environments. The clonal variation in adaptation to fungal endosymbionts may eventually lead to specialization and speciation of aphids.

INTRODUCTION

Evolution, specialisation and speciation depend on natural genetic and phenotypic variation of individuals in a population (Darwin 1859). In sexual species such natural variation is constantly created by recombination during sexual reproduction. In asexual species or species with only occasional sex, genetic variation is maintained too (Via & Lande 1985). Here, genotypic variation could result from differential performance of genotypes in response to variation in resources (e.g. host plants; Mitter *et al.* 1979; Mopper & Strauss 1998; Ferrari *et al.* 2006), natural enemies (Henter & Via 1995; Ferrari *et al.* 2001) or parasites (Haag & Ebert 2004). Individuals need certain traits to survive and perform well under specific local conditions and thus, different environments may favour different genotypes. This variation among genotypes in phenotypic sensitivity to different environments is referred to as genotype x environment interactions (G x E; Falconer 1952)

and observed for a variety of species, ranging from plants (Pederson 1968) to arthropods (Via 1984; Keller & Ross 1993; Vieira *et al.* 2000) and also humans (Humphries *et al.* 1995). The performance of one genotype in a specific environment can trade off with its performance in a different environment because beneficial changes in one trait are often linked to detrimental changes in another (Stearns 1989). These trade-offs prevent one genotype from getting fixed and replacing all other genotypes. For example, in bacteria an originally genetically homogenous culture can diversify rapidly in heterogeneous environments and trade-offs in the competitive ability of the new genotypes maintain this new genetic variation (Rainey & Travisano 1998). For polyphagous insects, such as certain aphid species that persist in asexual populations for most of their life cycle, clones performing well on one host plant species have been shown to perform badly on other host plant species (Via 1991; Ferrari *et al.* 2006).

Studies on genotype x environment interactions of phytophagous clonal arthropods mostly focused on environmental heterogeneity caused by the availability of different host plant species (Via 1991; Ferrari *et al.* 2006) or heterogeneity within one resource caused by different abiotic conditions such as light intensity (Service & Lenski 1982; Weider *et al.* 2005). However, heterogeneity within a plant species can be caused by the presence of symbiotic microorganisms such as mycorrhizal fungi and endosymbiotic fungi, both of which play a key role in structuring natural communities (van der Heijden *et al.* 1998; Omacini *et al.* 2001; Rudgers, Koslow & Clay 2004). Endophytic fungi are inconspicuous associates of almost all species of plants (Clay 2004) and live within the intercellular tissue of plants (Clay 1988). Endophytes of the genus *Neotyphodium* associate with cool-season grasses and are often asymptomatic and vertically transmitted via grass seeds. *Neotyphodium* produce alkaloids, which can increase the resistance of the plant against attacks by insect herbivores (Siegel *et al.* 1990; Bultman & Bell 2003; Meister *et al.* 2006). The increased resistance against herbivores can provide a competitive advantage for host plants when interacting with other plants (Clay & Holah 1999; Clay, Holah & Rudgers 2005). Moreover, the exact outcome of the symbiotic interaction between plant and fungus appears to be linked to the precise identity of plant and fungal genotype (Cheplick & Cho 2003). Likewise, not all herbivorous insects do respond negatively to endophytes. For example, the cereal aphid species *Metopolophium dirhodum* and *M. festucae* show no reduction in their total life span or fecundity when feeding on endophyte-infected plants (Meister *et al.* 2006; S. A. Härri, unpublished data). That the effects of endophytes on herbivores differ among species suggests that endophytes may mediate coexistence and

thus maintain species diversity among herbivores sharing the same resource (S. A. Härri, unpublished data).

For studies on genotype x environment interactions, aphids are useful model systems as they reproduce for most parts of their life cycle parthenogenetically (Dixon 1998). This allows to culture asexually reproducing clonal lines in the laboratory and to assign differences in clonal performance directly to genetic differences. Clonal variation in aphids has been demonstrated for the type of life cycle and life-history traits (Moran 1991; Dedryver *et al.* 2001; Vorburger 2005), susceptibility to entomopathogenic fungi (Ferrari *et al.* 2001; Stacey *et al.* 2003), susceptibility to parasitic wasps (Henter & Via 1995; Ferrari *et al.* 2001; Gwynn *et al.* 2005; S. von Burg, unpublished data), sensitivity to pesticides (Foster *et al.* 1997) and host plant utilisation (Mackenzie 1996; Vorburger, Sunnucks & Ward 2003; Ferrari *et al.* 2006). Therefore, we propose that clonal variation and variation in phenotypic performance could occur in response to feeding on endophyte-infected and endophyte-free plants.

In our study we address the question whether endophyte presence in the food resource affects different clones of a single aphid species differentially, which would suggest that inconspicuous fungal endosymbionts of plants can contribute to the maintenance of genotypic diversity of these asexually reproducing herbivores. We tested 37 aphid clones of the cereal aphid *Rhopalosiphum padi* L. on endophyte-infected and endophyte-free tall fescue (*Lolium arundinaceum* Schreb.) and compared their development time (aphids develop through 4 nymphal stages before reaching adulthood and maturity), lifetime fecundity and adult lifespan. With our experiment we addressed the following questions: *(1)* Does the presence of endophytes in the plant resource adversely affect life-history traits of all *R. padi* clones equally? *(2)* If there is genotype x environment (clone x endophyte) interaction, what life-history traits of *R. padi* show most variation? *(3)* Are there trade-offs for performances on endophyte-infected and endophyte-free plants?

MATERIAL AND METHODS

Material

Our study system consisted of 37 different clones of the common bird cherry-cereal aphid *R. padi*, tall fescue *L. arundinaceum* (former *Festuca arundinaceum*), a widespread agricultural grass, and its endosymbiotic fungus *Neotyphodium coenophialum* (Morgan-Jones & Gams) Glen, Bacon and Hanlin, which is known to produce loline alkaloids, ergovaline and peramine (Clay & Schardl 2002). *Rhopalosiphum padi* was shown

previously to be sensitive to endophyte presence with a reduced lifespan and reduced fecundity when feeding on endophyte-infected *L. perenne* L. (Meister *et al*. 2006). The seeds of *L. arundinaceum* (variety Georgia 5) were provided by Prof. Jonathan Newman, Guelph University, Canada. The seeds were either endophyte-free (E-) or endophyte-infected (E+) by AR542 ("novel endophyte" *N. coenophialum*). In December 2005, E– and E+ seeds were planted and kept in a greenhouse for five months. After this propagation period, the grass was cut and single plants were transferred to pots (! 25 cm), which were then transferred to an experimental garden at the University of Zürich, Switzerland. The endophyte infection was checked by staining leaf tissue and by diagnostic immunoblotting using the "Phytoscreen field tiller endophyte detection kit" (Agrinostics Ltd. Co., Watkinsville, USA). Ten plants of each E- and E+ for which the staining and the immunoblots showed identical results were randomly selected for the experiment. To ensure the availability of enough infected and uninfected grasses during winter 2006, one tiller per pot was transferred to a plant room (light:dark regime of 16:8 h).

The aphid species *R. padi* has a holocyclic life cycle in Switzerland with a sexual phase and overwintering of eggs on *Prunus* spp. and a parthenogenetic summer phase on numerous species of Poaceae (Blackman & Eastop 2006). Winged dispersal morphs are produced during the parthenogenetic phase in response to crowding or food shortage but these winged morphs usually have a reduced fecundity (Dixon 1998). To obtain 37 different *R. padi* clones, fundatrices (asexual females hatching from over-wintered, sexually produced eggs) or their first offspring were collected from *P. padus* across Switzerland between March and July 2006 (see Appendix Table A1). As the collected individuals derived from sexually produced, over-wintered eggs we assumed they were all different clones. Molecular analysis of some of the used clones confirmed this assumption (J. C. Simon, personal communication). Each clonal line was initiated by a single individual. The clonal cultures were maintained on *L. arundinaceum* (Barcel, a commercially available endophyte-free grass line) and kept in a climate chamber with a light:dark regime of 16:8 hours, at a constant temperature of 22° C and humidity of 60%. The aphid clones were in culture for approximately 20 generations before the experiment started.

Experimental set-up

Several life-history traits such as nymphal development time, adult life span and fecundity for each of the 37 clones were measured when feeding on E- or on E+ (= endophyte treatment). Each combination of endophyte treatment and clone was replicated ten times,

resulting in a total of 740 starting individuals (37 clones x 2 endophyte treatments x 10 replicates). Out of the 740 starting individuals (F0 adults), 125 were winged (E-: 68, E+: 57) and 615 wingless morphs (E-: 302, E+: 313). Due to shortage of wingless individuals, we had to use winged and wingless morphs that were assigned randomly over the two endophyte treatments.

To start the experiment, we placed one adult aphid of each clone (F0 adult) on a leaf cutting of *L. arundinaceum* in a Petri dish (Ø 55 mm) that was lined with a moist filter paper to avoid desiccation of the grass cutting. The cuttings were 2-4 cm long and came from either E- or E+ plants. The first nymph produced by these F0 adults was then transferred singly to a new Petri dish of the same treatment (F1 generation). Not all F0 adults did reproduce and thus, the starting number of F1 nymphs differed among clones (E-: 284, E+: 247). The F1 nymph was followed through to adulthood and its natural death. Each day, the nymphal stage (assessed by sheded skins), the presence of dead aphids and all offspring produced were recorded. All newly produced nymphs were removed daily from the Petri dishes. Grass cuttings were exchanged every 3 days and filter papers every 10 days.

The proportion of F0 adults reproducing was analysed with the "original data set" (37 clones, E-: 370, E+: 370). Nearly half of the F1 nymphs did not reach adulthood (n = 247). Therefore, some of the analyses for the F1 generation were done on the "full data set" (37 clones, E-: 284, E+: 247) and some on a "reduced data set" of clones, for which at least three F1 nymphs reached maturity per endophyte treatment (20 clones, E-: 112, E+: 76). Analyses on the proportion of F1 reaching maturity and the proportion of F1 individuals reproducing were done on the full data set. Analyses on nymphal development time, proportion of winged F1 adults, total number of offspring (fecundity) and adult lifespan of the F1-generation were done on the reduced data set. Adult lifespan and reproductive lifespan (= lifespan during which the individual reproduced) were highly correlated ($r = 0.84$, $df = 186$, $P < 0.0001$) and therefore we present only data for adult lifespan.

Statistical analyses

All calculations and analyses were done with the statistical software R (Version 2.5.0 for Macintosh). Means are given as means ± SE. Analyses of variance (ANOVA) or generalised linear models (GLM) were used to test for effects of clone, endophyte treatment and their interaction (G x E). The morph type of the individuals analysed was also included as a factor, except for the proportion of reaching adulthood and the proportion of F1

individuals reproducing because the presence of wings for nymphs dying at a younger age than the fourth nymphal instar cannot be recorded. Interactions of morph type with other factors were not included in the analyses as model comparison tests revealed no significantly increased fit of the models (Crawley 2002), except for the proportion of F0 adults reproducing where the model fit was increased by including the interaction of clone and wing production. For the proportion of F0 adult reproducing (yes/no), the proportion of F1 nymphs reaching adulthood (yes/no), the proportion of F1 reproducing (yes/no) and the proportion of winged F1 adults, a GLM with quasibinomial error structure was used to account for overdispersion (Crawley 2002). For the number of offspring produced by F1 a GLM with quasipoisson error structure for counts and for correction of overdispersion was applied (Crawley 2002). For the proportion of winged F1 adults, the morph type of the F0 adults was included as additional factor. Development time and adult lifespan were ln-transformed to meet the assumptions of normality and heteroscedasticity of the residuals and analysed with ANOVA.

Pearson's product-moment correlations on clone means were performed to test for trade-offs between performance on E- and E+. Each of these correlations was either based on the "full data set" or the "reduced data set", depending on the analysed life-history trait. For each clone, relative performances on E+ and E- were calculated by subtracting the "E+ clone mean" from the "E- clone mean" for each life-history trait, again either based on the "full data set" or the "reduced data set" depending on the life-history trait. For all the traits negative values indicate better performance on E+, except for development time where negative values indicate shorter development time on E-. Relative performances among traits were compared with Spearman rank correlations to test whether clones performing relatively well on E+ in relation to a certain trait also do so in relation to other traits.

RESULTS

Endophyte presence

Endophyte presence in the resource plant reduced the proportions of F0 adults reproducing with 77% reproducing on E- and only 67% on E+ (Table 1). In the F1 generation, the proportions reaching maturity (Fig. 1a) and the proportions reproducing (Fig. 1b) were decreased on E+. Endophyte infection also significantly prolonged nymphal development time (Fig. 1c), significantly reduced fecundity (Fig. 1e) and tended to reduce adult lifespan (Fig. 1f) for F1 individuals. The proportions of winged morphs were not affected by

endophyte infection (Fig. 1d). Overall, most clones performed better on E- than on E+ grasses (Fig. 1 & 2).

Clonal variation in life-history traits

We found significant among-clone variation for all the measured traits, except for the probability of F1 individuals to reach maturity that showed only a statistical trend for clonal variation ($P = 0.054$; Table 1).

Genotype x environment interaction

The interaction between the presence of endophytes and the clonal identity (G x E) was statistically significant for all fecundity-related traits (Table 1), such as the probability of F0 reproducing, the probability of F1 reproducing and the number of offspring produced by F1 individuals. G x E interactions were also found for the proportion of winged F1 adults. Despite clonal variation, no significant G x E interactions were detected for the other traits (Table 1), such as the probability of F1 to reach maturity, development time and adult lifespan. For all traits measured a few clones performed better on E+ than on E- plants (Fig. 2).

Winged morphs

Of all the wingless F0 adults 77% reproduced, whereas only 48% of the winged F0 adults reproduced. For the probability of F0 adults to reproduce, there was a significant clone x winged morph interaction ($F_{31,634} = 2.74$, $P < 0.001$). A few of the F1 nymphs reaching maturity were winged (E-: 28, E+: 17). These F1 winged morphs had with 10.62 ± 0.36 days a significantly longer development time than wingless morphs, which took an average of 8.02 ± 0.18 days to develop (Table 1). Winged F1 individuals had several additional disadvantages with a significantly reduced adult lifespan (winged: 4.82 ± 0.41 days, wingless: 10.96 ± 0.57 days) and fecundity (winged: 1.44 ± 0.91 offspring, wingless: 31.58 ± 1.74 offspring) compared to wingless morphs. However, these effects of winged morphs on nymphal development time, adult lifespan and fecundity did not differ among clones and endophyte infection (model comparisons, see M & M).

Trade-offs and comparison of ranks

We could not detect trade-offs (i.e. negative correlations) in the performance of the F1 generation feeding on E- and E+ for any of the life-history traits measured. All recorded

traits of performance on E- and on E+ were significantly positively correlated (Fig. 2). The spearman rank correlations between life history traits were neither significant for nymphal development time and fecundity (rho = -0.39, df = 18, $P = 0.091$) nor for nymphal development time and adult lifespan (rho = -0.27, df = 18, $P = 0.258$). However, clones with increased fecundity on E+ plants also lived longer on E+ (rho = 0.59, df = 18, $P = 0.007$; Fig. 3), which suggests that there were some superior clones with overall high performance on E+ that may represent an adaptation to feeding on inferior plant quality.

DISCUSSION

We showed that the phenotypic responses of aphids to the presence of a fungal endosymbiont associated with its food plant can vary significantly among clones of the same aphid species. Although endophyte presence had an overall negative effect on aphid performance, a few clones perform better on infected than on uninfected plants. The strong negative reaction of the majority of *R. padi* clones to the endophyte presence was consistent with earlier findings (Eichenseer & Dahlman 1992; Hunt & Newman 2005; Meister *et al.* 2006). However, these previous experiments did not control for clonal identity and were probably carried out with a mixture of clones or with one clone only. Our study is the first to observe significant genotype x environment interactions between aphid clones and endophytes, suggesting large differences in response to the presence of endophytes depending on the genetic background of herbivores. The lack of endophyte effects on *R. padi* populations in some field experiments (Krauss *et al.* 2007; S. A. Härri, unpublished data) may be explained by differences in clonal compositions according to endophyte infection. Better knowledge of the exact clonal composition of *R. padi* assemblages on plants in the field could demonstrate whether there is clone-specific colonisation of host plants that coincides with their ability to perform well on infected plants.

Trade-offs have been shown for aphid species feeding on different host plants and together with the strong clonal variation in host choice, this suggests ongoing processes of specialisation (Via 1991; Ferrari *et al.* 2006). Unexpectedly, we found no trade-offs between clonal performance on E+ and that on E- plants. Clones performing well in one environment tended to perform well in the other, while some clones were generally poor performers in both environments. However, the presence of significant clone x endophyte interactions for fecundity related traits implies that different clones have different fitness rankings on endophyte-infected and endophyte-free plants. Changes in fitness ranks have been shown to be sufficient for specialization to evolve (Fry 1996). As *R. padi* is a

polyphagous species feeding on different grass and cereal species, clones performing bad in both, the E- and E+ environment, may have fitness advantages on other host plant species or may show trade-offs in susceptibility to parasitoids and pathogens.

A further reason for the maintenance of clonal variation in life history traits and the strong performance of some clones are genotype-by genotype interactions (GxG). We did not experimentally vary or control the genotypes of the plant, the fungus or the symbiotic associations between plant and fungus. However, particularly suitable genetic combinations of plant genotype, fungal strain and aphid clone may exist. Genetic matching is described for the plant-fungus interaction (Cheplick & Cho 2003) and may exist also for the plant-fungus-aphid interactions. Effects of plant – fungal genotype interactions even translate to higher trophic levels. For example, *Microctonus hyperodae* a parasitoid of the Argentine stem weevil (*Listronotus bonariensis*) is differently affected by endophyte presence depending on the exact fungal strain (Bultman, McNeill & Goldson 2003). Such genotype interactions among the three players plant, fungus, aphid still await thorough experimental investigation.

Apart from superior clones on *L. arundinaceum*, there were a few clones that had a relatively higher fecundity and lifespan on E+ grasses. These clones may be in the process to adapt to endophyte-infected tall fescue and diverge from the other, less well adapted clones. Endophyte adapted clones may out-compete other clones when kept on endophyte-infected plants in a similar way to cereal aphid species that are tolerant to endophyte presence in the food plant. Experiments investigating clonal competition over several generations on plants that are endophyte-free, endophyte-infected or show mixed infection would indicate whether high performing aphid clones really do have competitive advantages over low performing clones. In more species rich insect food webs that include natural enemies of aphids, the clonal variation in susceptibility to aphid enemies may also impose a selective pressure on the plant-fungus-aphid interactions that could help maintaining clonal variation in life history performance. For example, the susceptibility to aphid predators, parasitoids, and pathogens vary among clones (Henter & Via 1995; Losey & Denno 1998; Ferrari *et al.* 2001). The presence of endophytic fungi does not only reduce the performance of herbivores but can also negatively affect higher trophic levels such as aphid predators (de Sassi, Müller & Krauss 2006) and parasitoids (Bultman *et al.* 1997; Omacini *et al.* 2001; S. A. Härri, unpublished data). To understand these more complex interactions among plants, endophytes and natural enemies for herbivore populations and how these interactions affect the performance and life history trade-offs of aphid genotypes,

future studies must consider the genetic backgrounds of the players. For example, whether aphid clones that are well adapted to feed on a particular plant-endophyte association are better or worse than non-adapted aphid clones at defending natural enemies remains to be tested.

To conclude, our study demonstrated *(1)* that the presence of endophytes in *L. arundinaceum* generally reduced the fitness of most *R. padi* genotypes but there were a few aphid clones that performed exceptionally well on endophyte-infected plants; *(2)* that there were significant G x E interactions for fitness related traits; and *(3)* no trade-offs existed between the performance on endophyte infected versus endophyte free plants. The absence of trade-offs between the two environments suggests that selective forces other than endophytes help maintaining clonal variation in life history performance.

ACKNOWLEDGMENTS

We would like to thank Jochen Krauss and Tobias Züst for staining and immunoblotting of tillers, Anselme Fournier for general input on aphid keeping, Jean-Christophe Simon for confirming clonal identity, Bernhard Schmid and Penelope Barnett for inputs on grammar, Siriporn Bieri, Daniel Trujillo and Theresa Karpati for helping counting the aphids and René Husi and Helmut Brandl for assistance in the laboratory. This research was financed by a grant from SNSF to Christine Müller (grant number 631-0659-50), the Institute of Environmental Sciences and by private funds.

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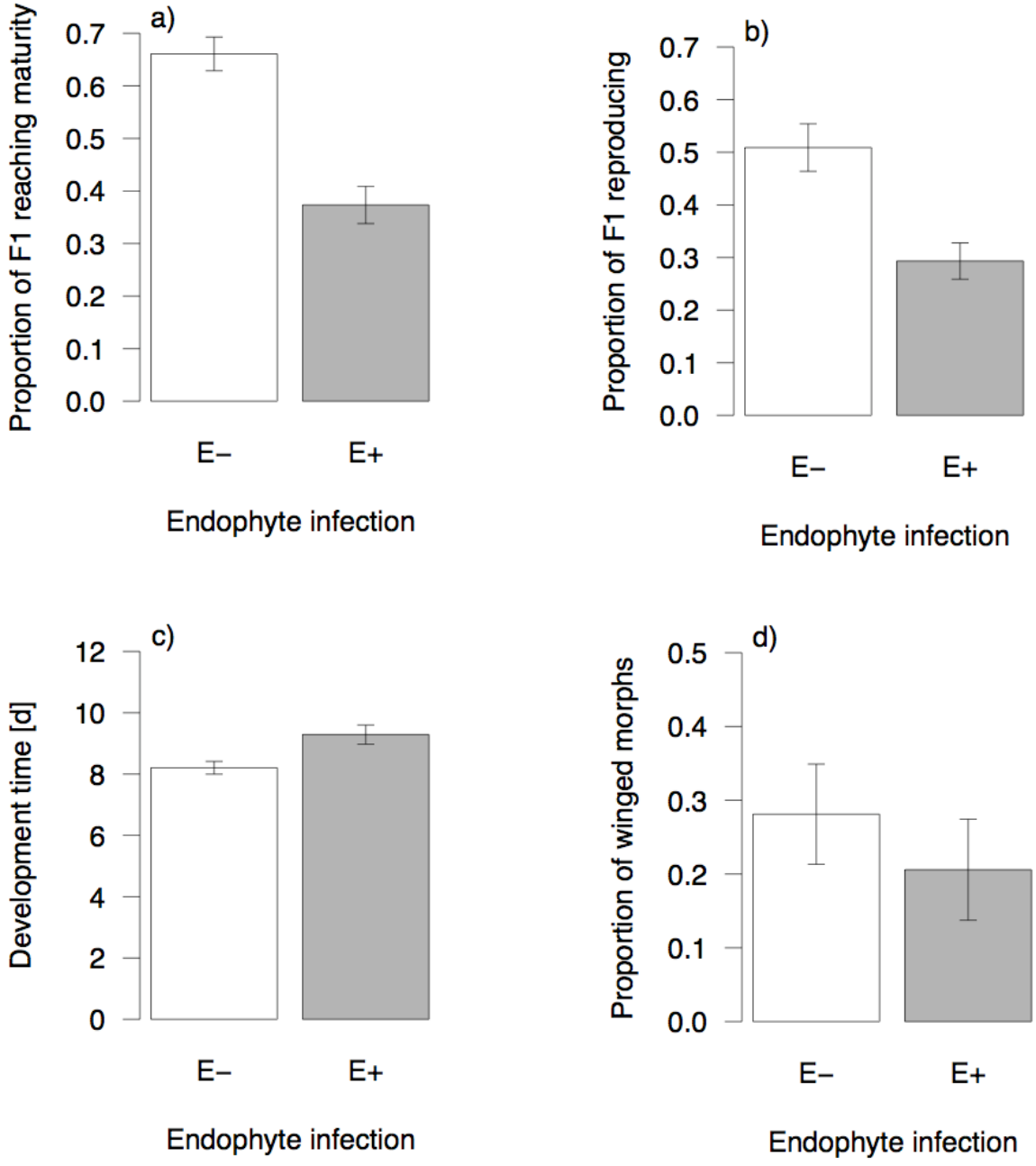
TABLES

TABLE 1. Test statistics for the life-history traits of *R. padi* clones measured with morph type (winged/wingless), clone identity, endophyte presence and the clone x endophyte interaction as explanatory variables. The traits measured were the probability of the F0 adults reproducing (yes/no), the probability of F1 individuals reaching maturity (yes/no), the probability of F1 adults reproducing (yes/no) and for the F1 generation nymphal development time (= time from birth to maturity), morph type of F1 generation (winged or wingless), lifespan (= adult lifespan in days) and fecundity (= total number of offspring produced). F1 nymphs reaching adulthood and F1 adults reproducing were calculated for the "full data set" including all 37 clones (E-: n = 284, E+: n = 247), while all other traits were calculated for the "reduced data set" with 20 clones (E-: n = 112, E+: n = 76). For the probability of F0 adults reproducing, the clone x morph interaction was additionally included into the analyses (see text), for all the other analyses, including morph interactions did not improve the model fit (Crawley 2002). When F0 adults were winged, the proportion of F1 adults being winged decreased (estimates: -1.47 ± 0.90; $F_{1,147} = 12.31$, P = 0.0006).

	Morph type	Clone identity	Endophyte	Clone x endophyte
Proportion of F0 adult reproducing	$\mathbf{F_{1,634} = 43.75}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{36,634} = 2.17}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{1,634} = 14.09}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{36,634} = 2.93}$ $\mathbf{P < 0.001}$
Proportion of F1 reaching maturity	NA	$F_{36,457} = 1.43$ $P = 0.054$	$\mathbf{F_{1,457} = 43.33}$ $\mathbf{P < 0.0001}$	$F_{36,457} = 0.45$ $P = 0.453$
Proportion of F1 reproducing	NA	$\mathbf{F_{36,457} = 2.23}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{1,457} = 25.13}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{36,457} = 1.70}$ $\mathbf{P = 0.008}$
Development time	$\mathbf{F_{1,147} = 71.84}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{19,147} = 5.01}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{1,147} = 11.37}$ $\mathbf{P = 0.001}$	$F_{19,147} = 1.45$ $P = 0.112$
Proportion of winged morphs	NA	$\mathbf{F_{19,147} = 6.32}$ $\mathbf{P < 0.0001}$	$F_{1,147} = 3.00$ $P = 0.086$	$\mathbf{F_{19,147} = 1.68}$ $\mathbf{P = 0.046}$
Fecundity	$\mathbf{F_{1,147} = 164.58}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{19,147} = 3.22}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{1,147} = 6.25}$ $\mathbf{P = 0.013}$	$\mathbf{F_{19,147} = 1.74}$ $\mathbf{P = 0.036}$
Lifespan	$\mathbf{F_{1,147} = 32.66}$ $\mathbf{P < 0.0001}$	$\mathbf{F_{19,147} = 2.30}$ $\mathbf{P = 0.003}$	$F_{1,147} = 3.24$ $P = 0.074$	$F_{19,147} = 0.82$ $P = 0.682$

FIGURES


a)
Proportion of F1 reaching maturity
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
E–
E+
Endophyte infection
b)
Proportion of F1 reproducing
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
E–
E+
Endophyte infection
c)
Development time [d]
0
2
4
6
8
10
12
E–
E+
Endophyte infection
d)
Proportion of winged morphs
0.0
0.1
0.2
0.3
0.4
0.5
E–
E+
Endophyte infection

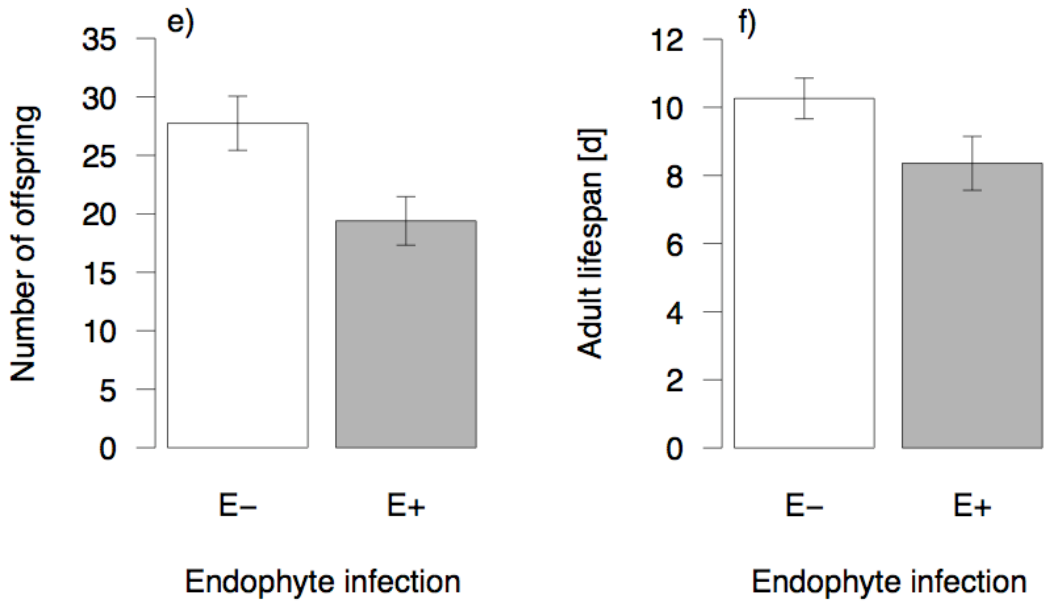


FIGURE 1. The mean performance of all clones of *R. padi* illustrated by a) the proportion of F1 nymphs reaching maturity, b) the proportion of F1 generation reproducing, c) the nymphal development time of the F1 generation, d) the proportion of winged F1 adults, e) the number of offspring (= lifetime fecundity) and f) the adult lifespan of the F1 generation on endophyte-free (E-, white bars) and endophyte-infected (E+, grey bars) *L. arundinaceum*. Statistical values can be found in Table 1, whereby a) and b) were analysed for the "full data set" (37 clones, E-: n = 284, E+: n = 247) and c), d), e) and f) for the "reduced data set" (20 clones, E-: n = 112, E+: n = 76). Bars refer to ± 1 SE.

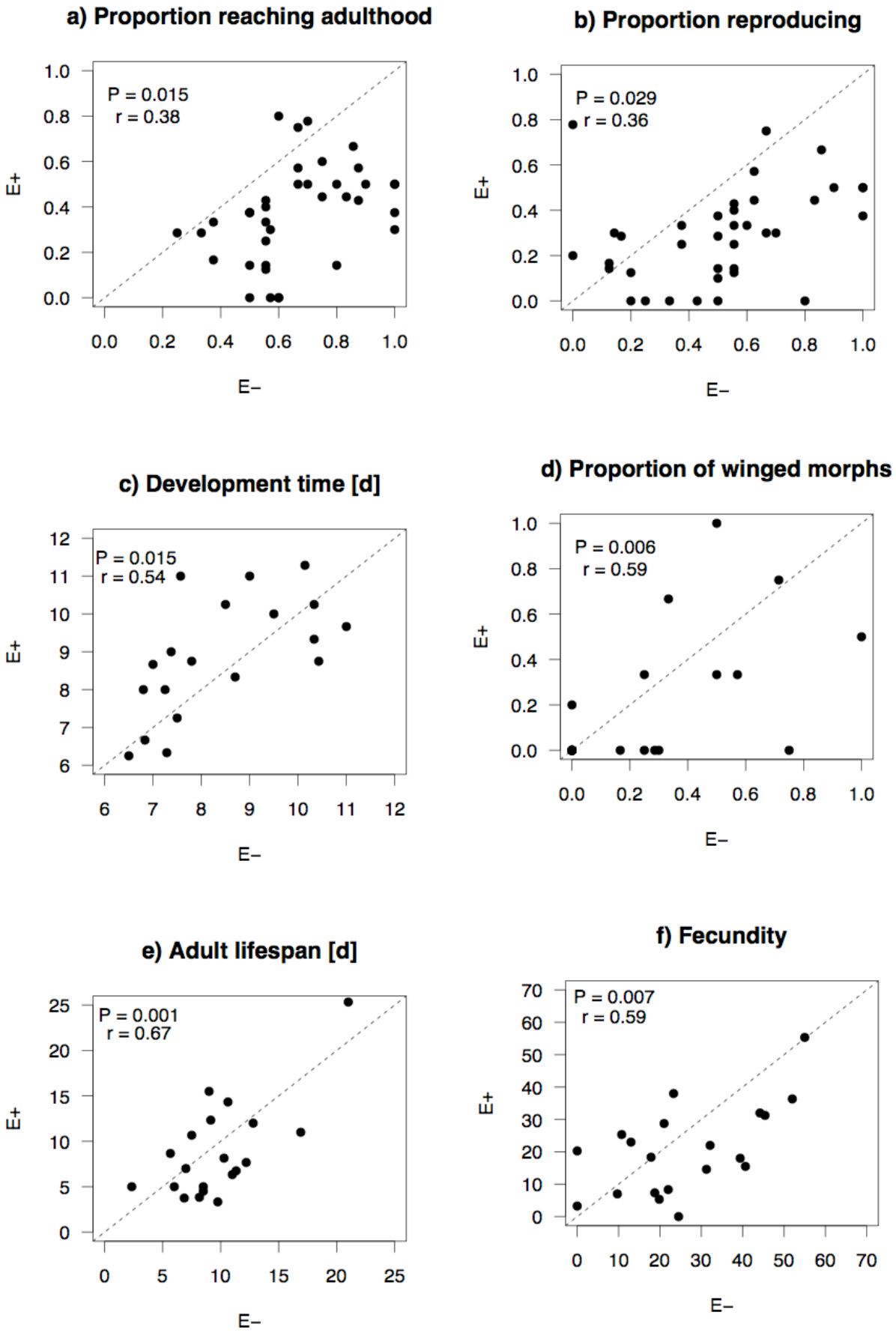

a) Proportion reaching adulthood
P = 0.015
r = 0.38
b) Proportion reproducing
P = 0.029
r = 0.36
c) Development time [d]
P = 0.015
r = 0.54
d) Proportion of winged morphs
P = 0.006
r = 0.59
e) Adult lifespan [d]
P = 0.001
r = 0.67
f) Fecundity
P = 0.007
r = 0.59
E+
E−

FIGURE 2. Mean clonal performance on E- plants plotted against mean clonal performance on E+ plants for a) the proportion of F1 nymphs reaching adulthood, b) the proportion of F1 adults of reproducing, c) nymphal development time, d) the proportion of winged morphs, e) lifetime fecundity and f) adult lifespan. The dashed line represents of the values where each clone performs equally on E- and E+ plants. Points above the dashed line represent clones performing better on E+ whereas points below the line represent clones performing better on E-, except for c) development time where the opposite is true (shorter developmental time is assumed to be advantageous). The values of the Pearson's product – moment correlation are written in each panel. a) and b) were calculated for the "full data set" (37 clones, E-: n = 284, E+: n = 247); c), d), e) and f) were calculated for the "reduced data set" (20 clones, E-: n = 112, E+: n = 76).

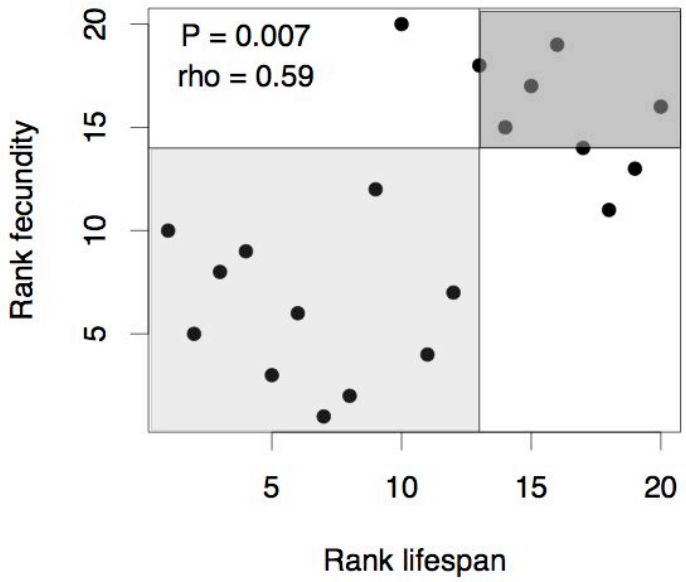


FIGURE 3. Correlation between ranks of the relative performance for adult lifespan *versus* fecundity. The best performing clones on E- have the lowest ranks while the best performing clones have the highest ranks (1 to 20). The lines represent the thresholds above which clones on E+ plants performed relatively better than clones to E- plants. The light grey area represents the values within which clones on E- outperform clones on E+ for both life-history traits. The dark grey area represents the area where clones performed better on E+ for both life-history traits. The Spearman correlation of life span and fecundity was significantly positive and was calculated for the "reduced data set" (20 clones, E-: n = 112, E+: n = 76).

APPENDIX

TABLE A1. List of the 37 clones used in our experiment, their sampling location and sampling date. The clones that were used for analyses on the "reduced data set" are in bold.

clone name	No.	sampling location	x-coordinate1	y-coordinate1	sampling date
ALPEN	**1**	**Thun**	**614500**	**178100**	**05/05/06**
APPLE	**2**	**Rohrschach**	**758100**	**260900**	**25/04/06**
BLACK	3	Galgenen	707500	226500	27/04/06
BODHI	**4**	**Schmidrüti**	**710800**	**252800**	**17/05/06**
CUCOO	5	Kreuzlingen	728500	280500	26/04/06
CYCLE	6	Monthey	563800	124000	08/05/06
FALLS	**7**	**Berschis**	**744600**	**219200**	**27/04/06**
FELSA	8	Horw	667700	208100	04/05/06
FLAGS	9	Bischofszell	735500	262300	15/05/06
GOOSE	**10**	**Davos**	**784100**	**187300**	**05/06/06**
HALLE	**11**	**Hard, Birsfelden**	**615000**	**266400**	**10/04/06**
HARRY	**13**	**Niederneunforn**	**700800**	**272000**	**15/05/06**
HINGI	**14**	**Martina**	**830100**	**196400**	**05/06/06**
HOMER	15	Zürich	683400	250100	14/04/06
HOTTY	**16**	**Aarwangen**	**624400**	**233000**	**02/05/06**
KLETT	17	Trasadingen	675100	281200	29/04/06
LONLY	19	Lucens	554700	172800	09/05/06
LUCER	**20**	**Meyrin**	**495600**	**120600**	**03/05/06**
MASSA	21	Langenthal	624900	229700	10/05/06
MEISE	22	Weinfelden	724400	269500	15/05/06
NOISE	**24**	**Thalwil**	**684500**	**238600**	**27/04/06**
ODEGA	**25**	**St. Blaise**	**566100**	**206600**	**02/05/06**
OLDER	**26**	**Bülach**	**682900**	**267100**	**15/05/06**
PILOW	27	Sarnen	662100	194200	04/05/06
POLAR	**28**	**Salavaux**	**569100**	**195800**	**10/05/06**
RHINO	29	Laufen	688100	281100	12/04/06
RUINS	**30**	**Casaccia**	**771900**	**135800**	**04/06/06**
SATIN	31	Mézières	547200	160600	09/05/06
SHAME	**32**	**Cham**	**676900**	**227900**	**04/05/06**
SHOOT	33	Disentis	708500	172900	27/04/06
SIXTY	**34**	**St. Loretto**	**724400**	**244300**	**25/04/06**
SMELL	35	Tiefencastel	763700	170000	28/04/06
SOLID	**36**	**Saxon**	**577500**	**110600**	**09/05/06**
SPARK	**37**	**Tinizong**	**767500**	**160700**	**03/06/06**
TASHI	38	Moosseedorf	603500	207300	10/05/06
TULIP	39	Grenchen	599200	227200	02/05/06
WOODS	**40**	**Gumefens**	**572800**	**169700**	**03/05/06**

[1] In the Swiss coordinate system.

CHAPTER 6

"Nothing in biology makes sense except in the light of evolution."

THEODOSIUS DOBZHANSKY

Can aphids learn to cope with the presence of endophytic fungi in their food plants?

S.A. HÄRRI [1], J.KRAUSS [1] and C.B. MÜLLER [1]
[1] *Institute of Environmental Sciences, University of Zürich, Winterthurerstrasse 190, CH - 8057 Zürich, Switzerland*
shaerri@uwinst.unizh.ch

Abstract

Plant-endophyte associations can have major impacts on the dynamics of consumer interaction-webs but long-term effects of mycotoxins and the ability of herbivores to adapt to these toxins have not been studied. To understand the potential of aphids to cope with mycotoxins, we compared the life-history parameters for aphids conditioned for several generations on endophyte-infected plants with those of endophyte-naïve aphids on both endophyte-infected and endophyte-free grasses. Aphids conditioned on endophyte-infected plants produced more offspring during the first days of adulthood than endophyte-naïve aphids independent of the endophyte infection of the test environment. However, the endophyte-conditioned aphids tended to have a shorter lifespan, which resulted in similar numbers of total offspring produced for endophyte-conditioned and endophyte-naïve aphids. The difference in life-history parameters caused by the conditioning environment suggests that the effects of endophytes on herbivore life-history traits may represent an adaptive change that should be considered in future studies of endophyte-herbivore interactions.
Keywords: *Rhopalosiphum padi, Neotyphodium lolii, Lolium perenne,* common strain, adaptation, long-term effects, microbes, endosymbionts, reproductive strategy, life-history traits

Introduction

Endophytic fungi live in close association with almost all species of plants (Arnold *et al.* 2000). However, most studies are conducted with agronomic grass systems (Saikkonen *et al.* 2006) where the association between plants and fungus results in the production of invertebrate and vertebrate toxic alkaloids (Clay 1990). Through these toxins the fungus often enhances resistance of plants against herbivores (Breen 1994; Clay & Schardl 2002; Müller & Krauss 2005; Omacini *et al.* 2001). Existing studies focus on short-term effects of endophyte presence using endophyte-naïve herbivores for their tests. Especially short lived and fast reproducing herbivore species may show life-history changes in the presence of the mycotoxins over just a few generations.

Aphids are among the most important pests and populations grow quickly through parthenogenetic reproduction. The lack of genetic recombination in parthenogenetic reproduction does not prevent aphids from adapting to new conditions (Blackman 1981; Loxdale & Lushai 2003). Individuals with an advantageous mutation can pass it to all their descendants and a mutation can spread rapidly through populations (Dixon 1998). The "telescoping" of generations allow aphid embryos to get in contact with, and physiologically react to, any ingested material that passes the haemolymph of their mothers (Via 1991).

The cereal aphid *Rhopalosiphum padi* has shown poor reproduction and survival on the agricultural grass *Lolium perenne* infected with the common endophyte, *Neotyphodium lolii*, in a short-term experiment (Meister *et al.* 2006). However, if aphids experiencing the endophyte for several generations adapt in some way to the endophyte, we would expect aphids conditioned on endophyte-infected grasses to perform differently on endophyte-infected grasses than aphids naïve to endophytes.

Methods

To test how individual aphids react to the presence of endophytes on a longer time scale, aphids reared on endophyte-infected plants for several generations were compared with endophyte-naïve aphids. The stock culture of the aphid *Rhopalosiphum padi* was started with a few individuals collected near the University of Zürich, Switzerland in May 2003. The culture was kept in a controlled temperature room at 20°C and 16:8 h light:dark cycle on *L. perenne* cv. ARION, a commercially available endophyte-free fodder grass (staining of 30 seeds: 0% infection) provided by FAL Reckenholz, Switzerland.

The perennial ryegrass *Lolium perenne* used in the experiment was the New Zealand cultivar Samson, which was either endophyte-free (E -: identity number A 11104) or endophyte-infected by the common strain of *Neotyphodium lolii* (E +: identity number A12038) and was provided by Brian Tapper, AgResearch, NZ. The endophyte status was assessed (1) by staining and microscopic examination of 30 seeds of E- and 30 seeds of E+, and (2) by immunoblot assays ("Phytoscreen field tiller endophyte detection kit" by Agrinostics Ltd. Co. (http://www.agrinostics.com/)) of 120 plants of E- and 120 plants of E+ with a minimum age of 2 weeks grown in the greenhouse. The microscopic staining revealed an infection level of 0% for E- and 93% for E+. The immunoblot assays showed an infection level of 0.008% for E- and 85% for E+.

Before the start of the life-history experiment, the aphids were conditioned in Petri dishes on cuttings of either E- or E+ *L. perenne* for 2 months, which corresponds to approximately 10 aphid generations (further referred to as "conditioning environment"). The Petri dishes were lined with moist filter paper and the cuttings were replaced every second day. The plants providing the cuttings were planted at the beginning of the conditioning phase in a 30 cm x 20 cm seed tray (approximately 1000 seeds). One seed tray was planted with E- seeds, one with E+ seeds. The cuttings used were a random mixture of upper leaf blades and from the lower leaves, the sheath, stem and blade. At the end of the conditioning period, 3rd or 4th instar nymphs were tested in a life-history experiment: aphids conditioned on E- were tested on E- and E+ and aphids conditioned on E+ were tested on E- and E+ (further referred to as "test environment"). Each treatment combination was replicated ten times. Each 3rd or 4th instar nymph was transferred individually into one Petri dish with a wet filter paper and cuttings of either E- or E+ and was followed over the entire lifespan. As life-history parameters, we recorded life-time reproductive success (divided into number of nymphs produced during the first 6 days of adulthood, number of nymphs produced between day 6 and day 11 of adulthood and number of nymphs produced between day 11 and death), adult longevity and developmental time from birth to adulthood for the first one to three nymphs produced by each mother.

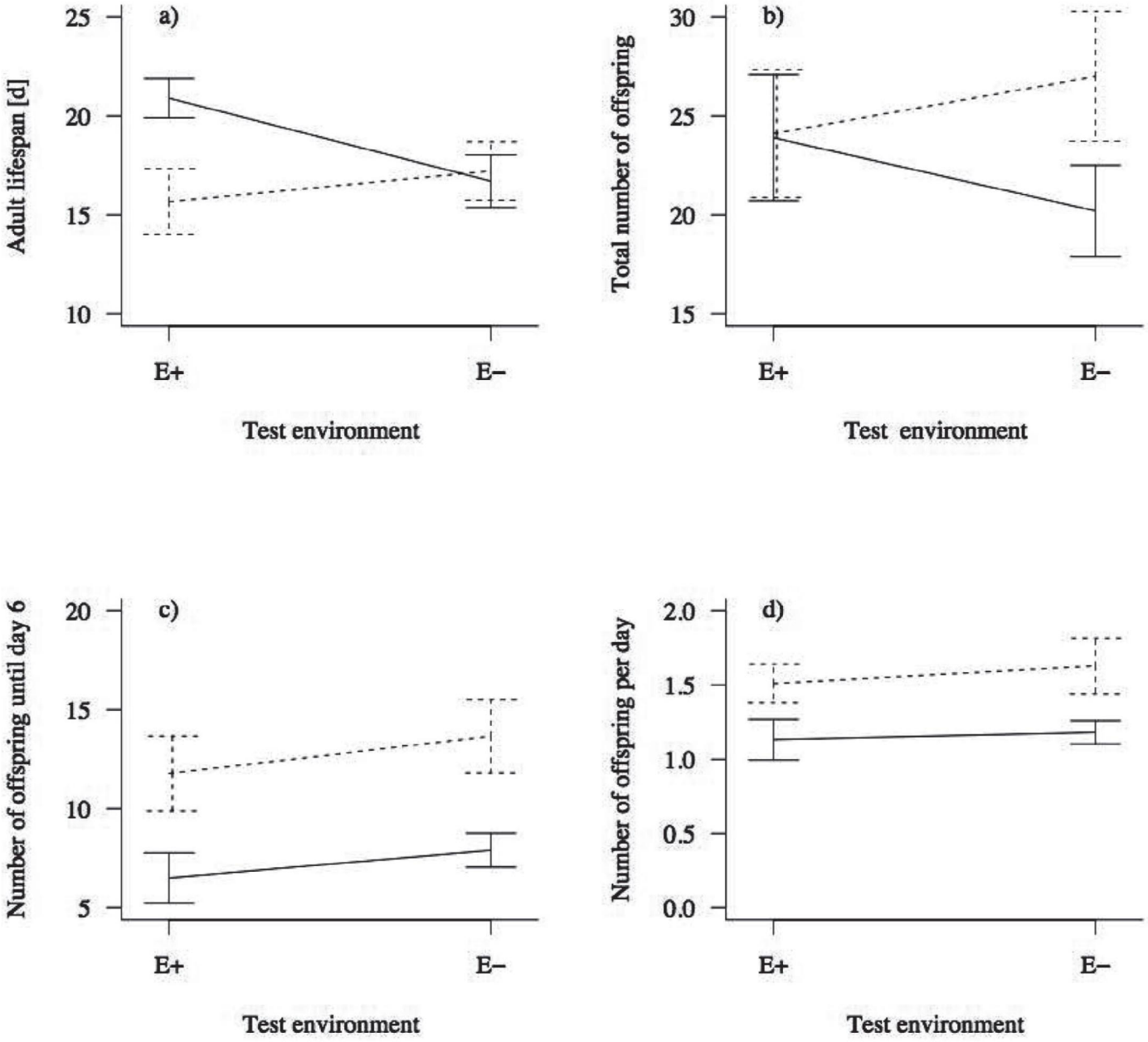
All life-history parameters were analysed separately using two-way ANOVA with the explanatory variables being infection of conditioning environment and infection of test environment and their interaction.

Results

The different life-history parameters measured showed different responses to the conditioning of aphids on endophyte-infected plants. Adult lifespan was affected by an interaction of the conditioning environment and the test environment, with aphids living the longest on E- when conditioned on E- ($F_{1, 34(interaction)}$=4.40, P=0.043; Fig. 1a) but the test environment and the conditioning environment itself did not affect adult lifespan ($F_{1, 34 (test)}$=1.16, P=0.290; $F_{1, 34 (conditioning)}$=2.95, P=0.095; Fig. 1a). The total number of offspring produced was neither affected by the test nor the conditioning environment, nor the interaction ($F_{1, 34 (test)}$=0.04, P=0.848; $F_{1, 34 (conditioning)}$=1.36, P=0.252; $F_{1, 34 (interaction)}$=1.20, P=0.281; Fig.1b). However, the number of offspring produced during the first 6 days of adulthood was significantly higher for aphids conditioned on E+ independent of the test environment ($F_{1, 34 (test)}$=1.20, P=0.282; $F_{1, 34 (conditioning)}$=13.66, P=0.0008; $F_{1, 34 (interaction)}$=0.03, P=0.871; Fig.1c). In contrast, the number of offspring produced between day 6 and day 11 of adulthood was neither affected by the test nor the conditioning environment or the interaction ($F_{1, 34 (test)}$=0.16, P=0.688; $F_{1, 34 (conditioning)}$=1.39, P=0.246; $F_{1, 34 (interaction)}$=0.47, P=0.495). The same was true for the number of offspring produced between day 11 and death ($F_{1, 34 (test)}$=1.10 P=0.303; $F_{1, 34 (conditioning)}$=0.21 P=0.645; $F_{1, 34 (interaction)}$=1.86, P=0.182). To integrate lifespan and fecundity, we calculated the number of offspring produced per day. Aphids conditioned on E+ produced overall more offspring per day than those on E-, independent of the test environment ($F_{1, 34 (test)}$=0.35, P=0.557; $F_{1, 34 (conditioning)}$=9.11, P=0.005; $F_{1, 34 (interaction)}$=0.06, P=0.808; Fig. 1d).

The time of development to adulthood for the one to three first emerged nymphs was neither affected by the test environment ($F_{1, 33}$=1.78, P=0.191) nor by the interaction of test and conditioning

Figure 1 The life-history parameters measured of *Rhopalosiphum padi* in the test environment on either endophyte-infected (E+) or endophyte-free (E-) *Lolium perenne*. The solid lines represent aphids conditioned on E- plants and the dashed lines represent aphids conditioned on E+ plants. Presented are means ± s.e. for a) adult lifespan, b) total number of offspring produced, c) number of offspring produced during the first 6 days of adulthood and d) number of offspring produced per day. For the results of the statistical analyses refer to the results section.



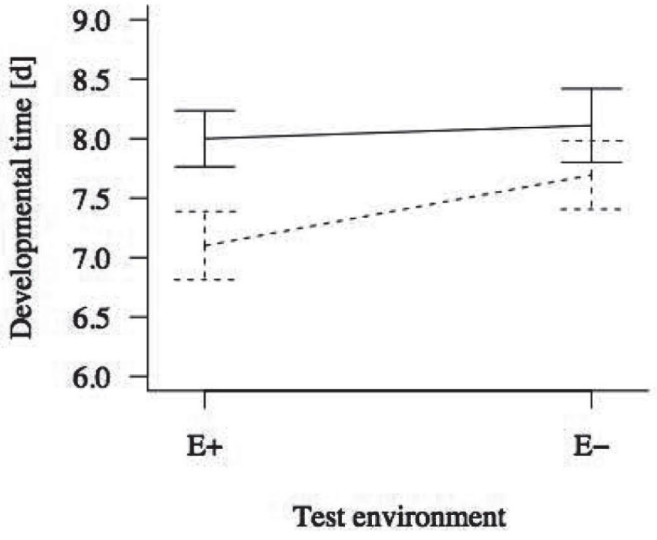
environment ($F_{1, 33}$=0.73, P=0.398), but was by the conditioning environment ($F_{1,33}$=5.55, P=0.024; Figure 2) with aphids conditioned on E+ developing significantly faster than aphids conditioned on E- .

Discussion

For all life-history parameters measured, the endophyte infection of the test environment did not have any influence. This is contrary to the observed changes in life-history traits of *R. padi* in several short-term experiments using endophyte-naïve aphids (e.g. Meister *et al.* 2006). However, the conditioning environment altered the expressed life-history traits: The exposure to endophyte infection over approximately 10 asexual generations appeared to trigger a change in the reproductive strategy. Aphids conditioned on E+ plants produced more offspring during the first few days of adulthood independent of the test environment. However, the total numbers of offspring produced over the entire lifespan remained the same for aphids conditioned either on E+ or on E-.

We speculated that aphids conditioned on endophyte-infected plants over several generations learn to cope with the presence of the mycotoxins and would somehow behave differently from endophyte-naïve aphids. Our results show that the conditioning influences reproduction in the first few days of adulthood, which is similar to 'fecundity compensation' that has been shown as a reaction to the presence of parasites (Minchella 1985; but see Krist & Lively 1998) or as a response in aphids to the presence of secondary parasitoids (van Veen *et al.* 2001) and could be a response to the shorter lifespan caused by the mycotoxin. However, to understand whether this is a general response of herbivores to mycotoxins, further experiments with different aphid clones and different herbivore species are needed. To compare changes in life-history traits for different clones exposed to the same environment would provide insights into the underlying mechanisms and would contrast between adaptation based on genetics or based on experience only (Ferrari *et al.* 2001; Via 1990).

Figure 2 The developmental time (time to adulthood) of the first nymphs produced by *Rhopalosiphum padi* in the test environment on either endophyte-infected (E+) or endophyte-free (E-) *Lolium perenne*. The solid lines represent mothers conditioned on E- plants and the dashed lines represent mothers conditioned on E+ plants. For the results of the statistical analyses refer to the results section.



The influence of the conditioning environment on aphid performance has been observed before for the aphid *Acyrthosiphon pisum*. This species occurs on different host plants and clonal specialisation expressed in different life-history traits on the different host plants has been observed. The specialised host performance does not change after three generations on the alternate host indicating that in this case the host plant experience has a strong influence on aphid life-history traits (Ferrari *et al.* 2006; Via 1991).

The effect of endophytic fungi in *L. perenne* on *R. padi* shows a difference between laboratory experiments and field experiments. In field experiments we found no or little effects of *N. lolii* on *R. padi* (Krauss *et al.* 2007; Krauss *et al.* submitted). One reason for these results may have been the low levels of peramine measured in these field experiments. Another very speculative reason may be that the naturally colonising aphids in the field experiments are not endophyte-naïve and therefore, well adapted to the endophyte presence, whilst the aphids from laboratory cultures had adapted to a non-endophyte environment. In any case, more experiments and surveys of natural endophyte infections and associated herbivores occurring in the grasslands are needed.

We believe our study provides a first indication of the importance of studying effects of endophytes on herbivores over longer time scales to consider the possibility of evolutionary adaptation to the mycotoxins if we want to understand this interaction and its mechanisms in detail. This is especially important for the use of endophyte produced toxins as herbivore controls in applied agriculture as well as for basic ecological research on endophytes and their effects on insect food webs.

ACKNOWLEDGEMENTS

We thank Brian Tapper for providing the seeds, Julia Nüscheler for assistance in the lab and Christopher Philipson for comments on the manuscript. The study was financed by a grant from SNSF to CBM (631-065950).

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CHAPTER 7

"The system of life on this planet is so astoundingly complex that it was a long time before man even realised that it was a system at all and that it wasn't something that was just there"

DOUGLAS ADAMS

ENDOPHYTIC FUNGI MEDIATE COEXISTENCE BETWEEN HERBIVORE SPECIES

SIMONE A. HÄRRI & CHRISTINE B. MÜLLER

ABSTRACT

Different species of herbivorous insects often feed on the same food plant. Theoretically, coexistence is only possible if spatial or temporal refuges exist. Here, we show that coexistence of two herbivores on the same food plant can also be mediated by microbes. Fungal endosymbionts of plants produce alkaloids, to which herbivores are differentially susceptible. The competitive interactions between two species of herbivores lead to reversed results when plant endosymbionts are present, compared to the situation without endosymbionts. A trade-off between competitive ability and susceptibility to mycotoxins may explain herbivore coexistence in natural grasslands, where endosymbiont-infected and uninfected plants co-occur.

How can species dependent on the same resource coexist? According to theory, coexisting species must differ along at least one resource axis or the weaker competitor must have a refuge in space or time (Tilman & Pacala 1993). In addition, the presence of a trade-off between competitive abilities and predation susceptibility has been shown to lead to species coexistence (van Veen, van Holland & Godfray 2005). Here we show a mechanism for species coexistence based on the presence of endophytic fungi.

Endophytes are fungal plant endosymbionts, most commonly associated with cool-season grasses, which negatively affect plant quality for the plant consumers by producing alkaloids that are toxic to herbivores (Clay & Schardl 2002). Endophytes may differentially affect closely related herbivores of their host plants (Meister *et al.* 2006) and can lead to alterations of insect food webs associated with grass herbivores (Omacini *et al.* 2001). Here we show that an endophytic fungi commonly associated with English ryegrass can mediate coexistence of two common cereal aphids.

We used the fungus *Neotyphodium lolii*, a ryegrass-specific endophyte and two aphid species, *Rhopalosiphum padi* and *Metopolophium festucae.* We kept the aphid species either alone or in mixtures in small laboratory microcosms on either endophyte

infected or non-infected English ryegrass.

Pure populations of *R. padi* grew faster and reached higher population densities than pure populations of *M. festucae* on both infected and non-infected plants ($F_{1,56}$ = 49.38, $P < 0.001$). Endophyte infection on average had a negative effect on aphid numbers ($F_{1,56}$ = 18.13, $P < 0.001$), but as indicated by the significant interaction term in the analysis, this was largely due to a negative response of *R. padi* whereas *M. festucae* had similarly low densities on both infected and non-infected grasses ($F_{1,56}$ = 15.10, $P <$ 0.001). Therefore, we predicted that *R. padi* would outcompete *M. festucae* when grown together, irrespective of endophyte infection. However, when the two aphid species were kept together, *M. festucae* outcompeted *R. padi* in numbers on infected plants, while on non-infected plants the expected outcome occurred, with *R. padi* outcompeting *M. festucae* (Fig. 1).

Our results suggest a trade-off between population growth and resistance to endophyte toxins, benefiting the generally faster growing species *R. padi* on endophyte-free grass and of the more resistant *M. festucae* on endophyte-infected grass. Because in natural grasslands infected and non-infected grasses occur in a patchy mosaic (Clay & Schardl 2002) both aphid species can coexist without one outcompeting the other in the longer term. Our result demonstrates how minute, often overlooked microorganisms can have a decisive influence on herbivore species coexistence.

MATERIAL AND METHODS

To test for the effect of endophytes on the outcome of competition between the two cereal aphid species *Rhopalosiphum padi* and *Metopolophium festucae*, we performed a laboratory experiment. All the seeds of *Lolium perenne* used for the experiment belong to the same cultivar (infected seeds: 97 % infection, uninfected seeds: 9% infection).

In small microcosms, consisting of pots with *L. perenne* (Ø 10cm; 50 seeds) covered by a PET bottle with ventilation holes, we applied the following treatments, both on endophyte-infected and uninfected *L. perenne*: Low density mixtures (initial aphid densities: 5 adult *R. padi* and 5 adult *M. festucae*), high density mixtures (initial aphid densities: 10 adult *R. padi* and 10 adult *M. festucae*) and two single-species treatments, where each species was kept separate (initial aphid densities: 10 adults). Each of the treatment combinations was replicated 15 times. One week after sowing, the aphids were added to the microcosms. The 60 pots were kept in a climatic chamber (22ºC under a L:D 16:8 h photoperiod). After 27 days the experiment was terminated and we counted adults

and nymphs of each species.

Statistical analyses

Two separate analyses were done, one for the single-species treatments and one combining the single-species and mixture treatments. The factor endophyte infection was included in all analyses. Only the results for the total number of aphids (adults plus nymphs) are presented, as the results did not differ qualitatively for the separate analyses of number of adults, number of nymphs or the total number of aphids.

For the single-species treatment analysis, an ANOVA with the factors “aphid species”, “endophyte infection” and their interaction on the total number of aphids (ln [x + 1]-transformed) was performed.

For the combined analysis of single-species treatments and mixture treatments, the proportion of *R. padi* was calculated for each pot from the mixture treatments by dividing the total number of *R. padi* by the number of total aphids (number of *R. padi* + number of *M. festucae*). To get an estimate of the expected proportion and the variance in proportions of *R. padi* for the single-species treatments, each single-species treatment pot of *R. padi* was randomly assigned to a single-species treatment pot of *M. festucae* and the proportion of *R. padi* was calculated for these two randomly paired pots (number of *R. padi* divided by the number of *R. padi* + number of *M. festucae*). The proportions of *R. padi* were arcsine-square root-transformed and analysed using an ANOVA with the factors “aphid treatment” and “infection treatment”. The “aphid treatment” was split into a contrast "competition", comparing the two mixture treatments against the single treatment and a contrast "density" nested within "competition" to compare the high and low density mixtures. All statistical analyses were performed in R (version 2.2.1 for Mac OS X).

ACKNOWLEDGMENTS

We thank Bernhard Schmid, Jochen Krauss, Uli Steiner, Dennis Hansen and Jonathan Newman for comments on the manuscript and Brian Tapper for providing the seeds. The work was funded by the SNSF (Grant number 631-065950 to cbm).

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FIGURES

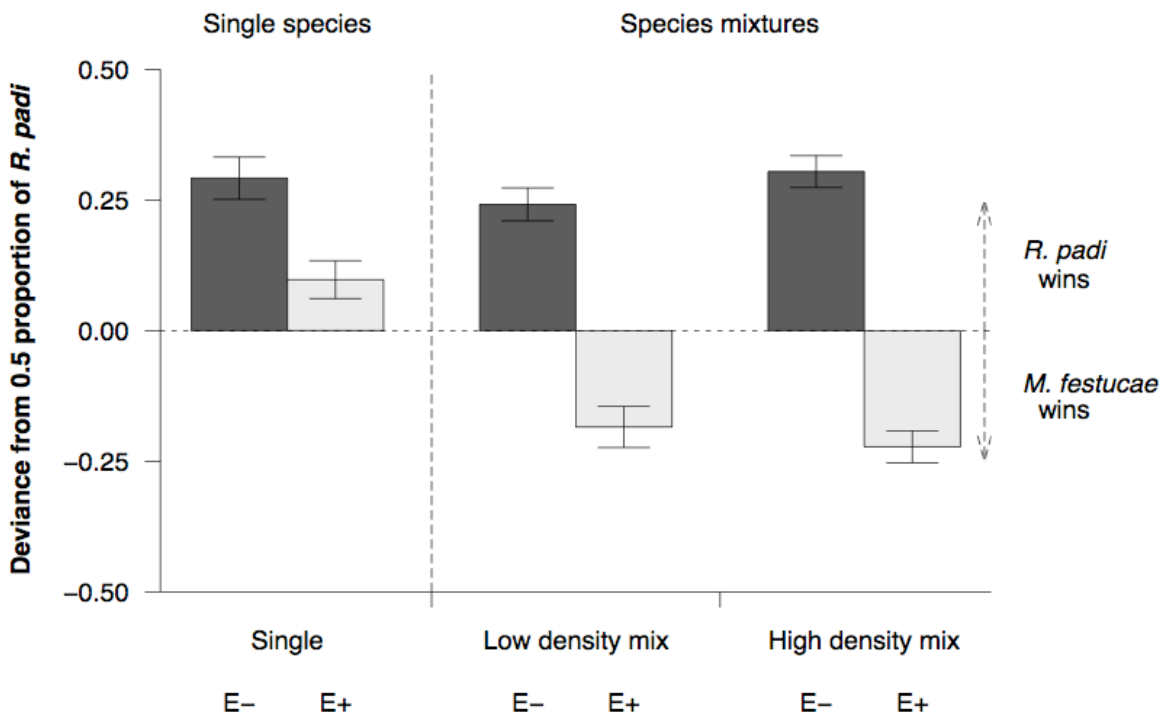
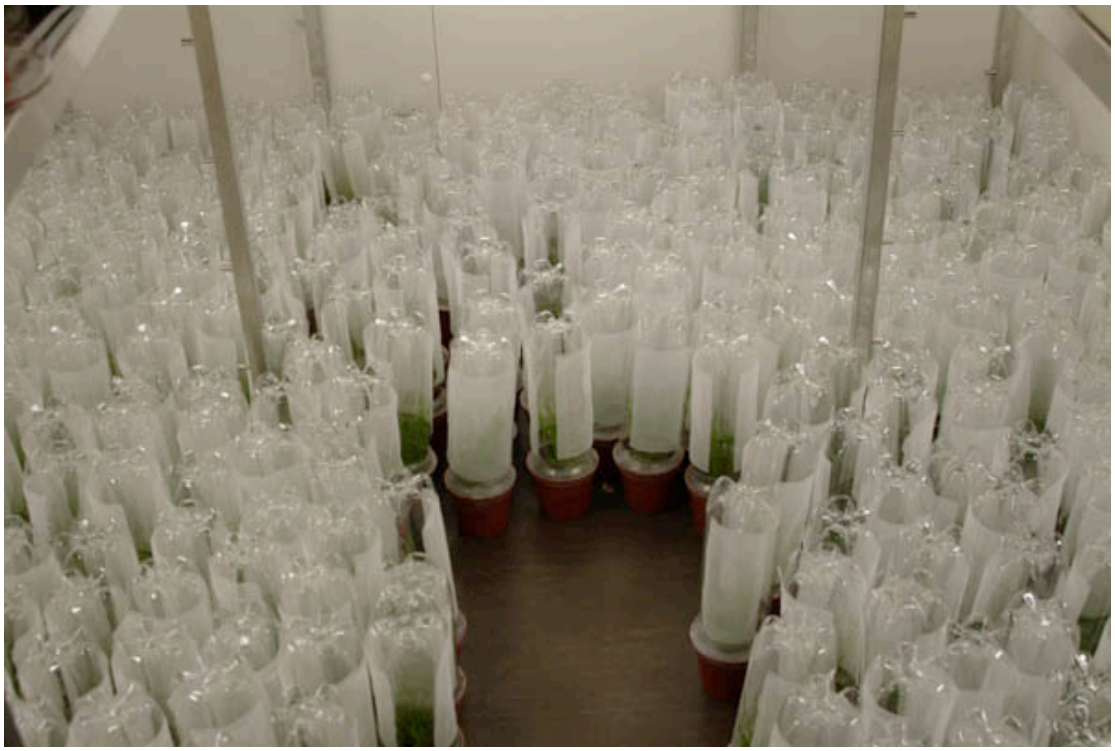


FIGURE 1. Deviance of equal population densities of two aphid species, *R. padi* and *M. festucae,* in dependence of endophyte infection (endophyte-free: E -, dark bars; and endophyte-infected: E +, light bars), competition (single species or species mixture) and initial densities (low, high). The horizontal dashed line represents equal number of individuals of both species. The bars show the deviance of *R. padi* proportions from equality. A positive deviance indicates a higher proportion of *R. padi* whereas a negative deviance indicates a higher proportion of *M. festucae*. The proportions of *R. padi* changed with endophyte infection ($F_{1,83} = 131.32$, $P < 0.0001$), competition (single species vs. mixture; $F_{1,83} = 20.00$, $P < 0.0001$) and with the interaction between infection and competition ($F_{1,83} = 18.08$, $P < 0.0001$). Initial density (treatments with mixtures) did not affect the outcome of the experiments ($F_{1,83} = 0.18$, $P = 0.676$).

CHAPTER 8



MICROBIAL SYMBIONTS AND PARASITOIDS FACILITATE COEXISTENCE OF HERBIVORES[1]

SIMONE A. HÄRRI & CHRISTINE B. MÜLLER

Although interspecific competition for resources may be viewed as an important factor for species interactions (Tilman & Pacala 1993), there is a role for natural enemies (Holt 1984) and microbial organisms (Hunter & Price 1992). Coexistence of two species with similar feeding modes on the same food resource is only possible if refuges in time or space for the weaker competitor exist (MacArthur & Levins 1964; Tilman & Pacala 1993). Plants commonly liaise with endosymbiotic fungi and the association leads to altered plant traits (Clay 1990). Grasses that harbor *Neotyphodium*-type endophytes are protected from damage by herbivores through the production of toxic alkaloids by the fungus (Clay 1988). In general, plants also get indirect protection from natural enemies of herbivores. Here, we demonstrate experimentally that species-specific differences in tolerance to toxins produced by fungal endosymbionts and in susceptibility to a shared parasitic wasp determine the relative abundances of two co-occurring herbivore species feeding on the same plant resource. Fungal endosymbionts provide a refuge from competition for the herbivore species tolerant to mycotoxins and further, reduce the effectiveness of the parasitic wasp. Independent of plant infection, the parasitic wasp attacks preferably the mycotoxin-tolerant herbivore species and thus, provides a refuge for the species sensitive to mycotoxins. Consequently, the shift in relative abundances caused by the presence of fungal endosymbionts is amplified through the action of the parasitic wasp. In a longer time frame, this shift in relative abundances might promote coexistence of two aphid species on perennial ryegrass stands that consist of endophyte-infected and uninfected plants, a pattern that is commonly observed in natural grasslands (Saikkonen *et al.* 2000; Müller & Krauss 2005).

How can species with similar feeding modes that depend on the same food resource coexist? According to theory, coexisting species must differ along at least one resource axis or the weaker competitors must have a refuge in space or time (MacArthur & Levins 1964; Tilman & Pacala 1993). In addition, the presence of a trade-off between

[1] to be submitted to *Nature*

competitive ability and susceptibility to predation may also lead to species coexistence (van Veen, van Holland & Godfray 2005). The role of resource competition in insect communities is assumed to be small compared to that in vertebrate herbivore communities (Lawton & Strong 1981). However, some studies argue that resource competition among insect species is important but occurs via subtle changes in plant nutritional quality rather than complete resource depletion (Denno, McClure & Ott 1995). We assume that aphids that plug into the phloem vessels of plants and coexist as species assemblages on the same plant species (Müller *et al.* 1999) may experience particularly strong resource competition.

Endophytic fungal symbionts are common associates of all species of plants but are particularly well studied in agricultural grasses (Saikkonen *et al.* 2006). For example, although perennial ryegrass *L. perenne* shows no clear phenotypic alterations when infected by its endophyte *Neotyphodium lolii* (Krauss *et al.* 2007*)*, its quality for herbivore consumers will be affected by the production of toxic alkaloids. The presence of endophytes can alter the food web structure of herbivores and their natural enemies (Omacini *et al.* 2001), ecosystem functioning (Rudgers, Koslow & Clay 2004) and plant succession (Rudgers *et al.* 2007). However, endophyte-produced toxins may differentially affect closely related herbivore species or even individuals of the same species (Faeth & Bultman 2002; Meister *et al.* 2006). Equally, endophyte-produced toxins can alter the performance of the natural enemies of herbivores further up the food chain. The presence of endophytes in grasses has been shown to impair the reproductive ability of aphid predators (de Sassi, Müller & Krauss 2006) and parasitic wasps (SAH, J. Krauss and CBM, manuscript in preparation). Parasitic wasps (= parasitoids) lay their eggs into nymphal instars of aphids and the developing larva feeds within the growing aphid, eventually mummifying and killing its host. The adult parasitoid emerges from the mummified aphid after metamorphosis (Godfray 1994).

We studied the combined effects of endophytes and a parasitoid that commonly attacks several species of cereal aphids on relative abundances of two herbivore species feeding on the same plant. In our experiment, we used the fungal endophyte *N. lolii* in association with *L. perenne*, the two species of cereal aphids, *Rhopalosiphum padi* and *Metopolophium festucae,* and the generalist aphid parasitoid *Aphidius ervi*. We kept the aphid species either alone or in mixtures in laboratory microcosm communities over a period of 12 weeks. The aphids were kept on endophyte-infected (E+) or endophyte-free (E-) *L. perenne* in the presence (P+) or absence (P-) of parasitoids.

When kept separately, the overall cumulative numbers of *Metopolophium* and *Rhopalosiphum* were not significantly different, which indicates similar growth rates and thus, similar competitive abilities for the two species (Fig. 1a and Fig. 1b). In the single species microcosms, the presence of endophytes decreased *Rhopalosiphum* densities while the presence of parasitoids reduced *Metopolophium* densities on endophyte-free plants. *Metopolophium festucae* showed higher densities on endophyte-infected plants than on endophyte-free plants when parasitoids were present (Fig. 1b). We therefore concluded that the two aphid species differ in their tolerance to endophyte presence, with only *Rhopalosiphum* showing strong negative effects. The two species also differed in their susceptibility to the parasitoid *Aphidius,* with *Rhopalosiphum* being more resistant to parasitoid attack (Fig. 1a). For *Metopolophium,* which was susceptible to parasitoids, the impact of parasitoids was reduced when feeding on endophyte-infected plants (Fig. 1b). The difference in susceptibility to the generalist parasitoid was also reflected in the small number of parasitoids persisting in the single species microcosms. The lowest parasitoid numbers were observed in both endophyte-free and endophyte-infected single-species microcosms of *Rhopalosiphum* while the highest numbers occurred in the endophyte-free single-species microcosms of *Metopolophium* and intermediate numbers in the endophyte-infected single *Metopolophium* treatment (Fig. 2).

In the mixed microcosms where both aphid species were kept together on either endophyte-free or endophyte-infected plants, parasitoids could choose which species to attack. The absolute cumulative number of aphids (*Rhopalosiphum* + *Metopolophium*) in the mixtures was not significantly affected by the presence of endophytes or that of parasitoids (Fig. 3). However, in the mixtures endophytes had a very strong effect on the relative numbers of the two aphid species. Where endophytes were present, *Metopolophium* was more abundant, whereas on uninfected plants *Rhopalosiphum* was more abundant. The presence of parasitoids slightly increases abundance of *Rhopalosiphum* independent of endophyte presence (Fig. 3 & Fig. 4). This must have been caused by the higher parasitoid attack rates on *Metopolophium*. The lack of an interaction between endophyte and parasitoid presence in affecting relative species abundances coincided with similar numbers of parasitoids on endophyte-free and endophyte-infected plants in mixed microcosms (Fig. 2). On endophyte-infected plants, impact of parasitoids was reduced by the presence of endophytes and therefore parasitoids exert a lower pressure on *Metopolophium* than on endophyte-free plants. On endophyte-

free plants, the presence of parasitoids created a refuge for *Rhopalosiphum*, as only *Metopolophium* numbers were reduced by the wasps.

To summarize, the presence of endophytes shifted the patterns of relative abundances of two aphid species feeding on and competing for the same resource plant. This shift was caused by the differences between the two aphid species in their relative tolerance to the presence of endophytes. As a consequence, the higher tolerance towards mycotoxins provided a refuge for *Metopolophium* on endophyte-infected plants, while on endophyte-free plants *Rhopalosiphum* was the superior competitor. The difference in susceptibility to a shared parasitoid and the indirect negative effect of the endophyte on parasitoid reproduction amplified the observed pattern of relative abundance of the two species of aphid.

Our result demonstrates how microscopic, often overlooked microorganisms that live in symbiosis with plants can have decisive influences on the interactions between herbivore consumer species. The presence of these microbial grass endophytes may facilitate the coexistence of two common cereal aphid species in natural grasslands where infected and non-infected grasses co-occur in a patchy mosaic (Saikkonen *et al.* 2000; Müller & Krauss 2005). The presence of a generalist parasitic wasp can significantly amplify this facilitation for coexistence by endophytes. Thus, both, the symbiosis of plants and fungi and the presence of parasitoids of herbivores represent refuges for herbivore competitors and thus guarantee species coexistence in natural grasslands.

MATERIAL AND METHODS

All the seeds of *L. perenne* used in the experiment were the cultivar Samson wildtype (infected seeds: 97 % infection, uninfected seeds: 9% infection) and were provided by Brian Tapper (AgResearch, NZ). The infection status was confirmed for the whole seed batch with two methods: 1) staining of seeds and microscopic examination and 2) by immunoblotting of stem sections. The stock culture of *M. festucae* was started in summer 2005 with a few individuals collected from *L. perenne* near the University of Zürich, Switzerland and maintained on commercially available endophyte-free fodder grass *L. perenne* ARION (staining of 30 seeds: 0% infection; fenaco, Winterthur, Switzerland). The stock culture of *A. ervi* was started with 250 individuals bought from Andermatt Biocontrol AG, Switzerland and kept on *M. festucae* feeding on *L. perenne* ARION.

Our microcosms consisted of potted *L. perenne* grown in garden compost (pot Ø 10cm; 100 seeds; 7-day old) covered by a PET bottle with ventilation windows. We

applied the following treatments, both on endophyte-infected and uninfected *L. perenne*: aphid mixtures (initial aphid densities: 5 adults and 5 nymphs of *R. padi* and 5 adults and 5 nymphs of *M. festucae*) and two single-species treatments, whereby each species was kept separate (initial aphid densities: 10 adults plus 10 nymphs). To half of the mixtures and half of the single species microcosms, two one-day old females and two one-day old males of *A. ervi* were added after 14 days. Each of the treatment combination was replicated 10 times. The 120 pots were kept in a climatic chamber at 22ºC and a light:dark cycle of 16:8 hrs. The pots were followed over 12 weeks, which corresponds to approximately 12 aphid and 5 parasitoid generations. Every second week, the grass was replaced with a fresh pot of *L. perenne* (Ø 10cm; 100 seeds; 7-day old) and all the aphids and parasitoids (alive and as mummies) were transferred and counted.

Statistical analyses

For the analyses, the number of aphids and parasitoids were summed up for each replicate over the 6 sampling dates. Analyses of the time series revealed the same general patterns as the cumulative counts and are therefore not shown.

For the single-species treatment analysis, an ANOVA with the factors "aphid species", "endophyte infection", and "parasitoid presence" and their interaction on the cumulative number of aphids was performed. For the absolute numbers of aphids within the species-mixture treatment, the number of *R. padi* and *M. festucae* were summed, ln-transformed and analysed independent of species identity with "endophyte infection", "parasitoid presence" and their interaction as factors. For the relative abundance of each species within the mixtures, the proportion of *R. padi* was calculated for each pot from the mixture treatments by dividing the total number of *R. padi* by the number of total aphids (number of *R. padi* + number of *M. festucae*). The proportions of *R. padi* were arcsine-square root-transformed and analysed using an ANOVA with the factors "endophyte infection", "parasitoid presence" and their interaction. All statistical analyses were performed in R (version 2.5.0 for Mac OS X).

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FIGURES

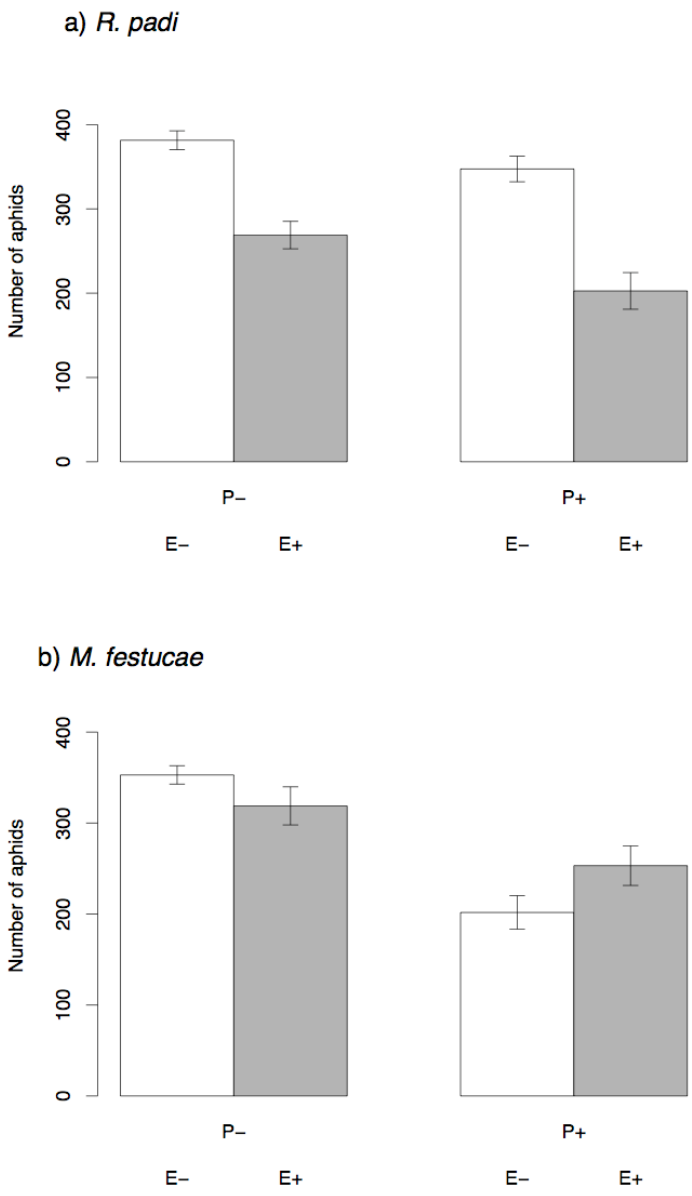

FIGURE 1. Aphid numbers in single species microcosms. Mean (± SE) of cumulative number of *R. padi* (a) and *M. festucae* (b) summed over 12 weeks. Each aphid species was kept alone on endophyte-free (E-) or endophyte-infected (E+) perennial ryegrass in the absence (P-) or presence (P+) of the generalist parasitoid *A. ervi*. The effect of endophyte ($F_{1,72}$ = 23.44, P < 0.001), parasitoid ($F_{1,72}$ = 41.08, P < 0.001), species x endophyte interaction ($F_{1,72}$ = 30.79, P < 0.001), species x parasitoid interaction ($F_{1,72}$ = 5.54, P = 0.021) and the three-way interaction ($F_{1,72}$ = 5.54, P = 0.021) were all statistically significant. The effect of species ($F_{1,72}$ = 2.23, P = 0.140) and endophyte x parasitoid interaction were not significant ($F_{1,72}$ = 1.16, P = 0.285).

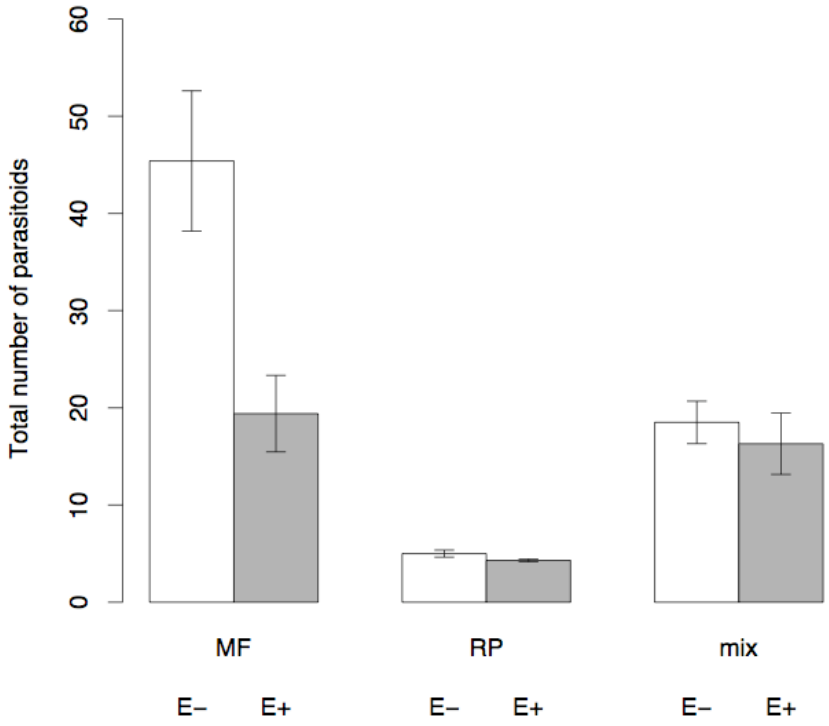

FIGURE 2. Parasitoid numbers. Mean cumulative number (± SE) of parasitoids (mummies plus adult parasitoids) over 12-weeks on the single species treatment with *M. festucae* (MF), the single species treatment with *R. padi* (RP) or the species mixture treatment (mix) on endophyte-free (E-) or endophyte-infected (E+) food plants. The effect of treatment ($F_{1,54} = 78.42$, $P < 0.001$), endophyte presence ($F_{1,54} = 13.86$, $P < 0.001$) and the treatment x endophyte interaction ($F_{1,54} = 4.62$, $P = 0.014$) were all statistically significant.

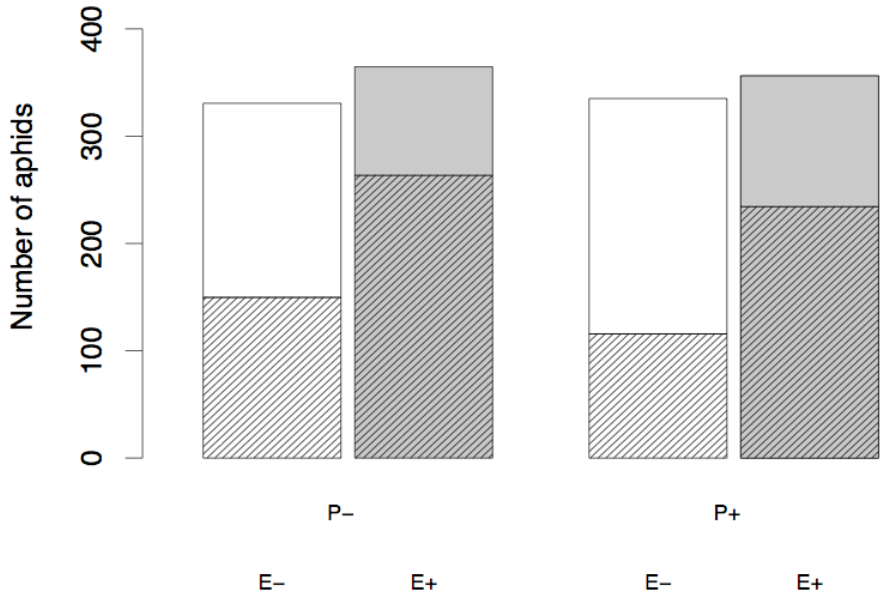

FIGURE 3. Aphid numbers in mixed microcosms. The bars show the mean absolute number of all aphids summed over 12-weeks on endophyte-free (E-) or endophyte-infected (E+) plants with (P-) or without (P+) the parasitoid *A. ervi*. Within each bar, the shaded part shows mean number of *M. festucae* and the clear part mean number *R. padi*. Total number of aphids were not influenced by endophyte ($F_{1,36} = 2.80$, $P = 0.103$), parasitoids ($F_{1,36} = 0.02$, $P = 0.877$), or the interaction ($F_{1,36} = 0.43$, $P = 0.516$).

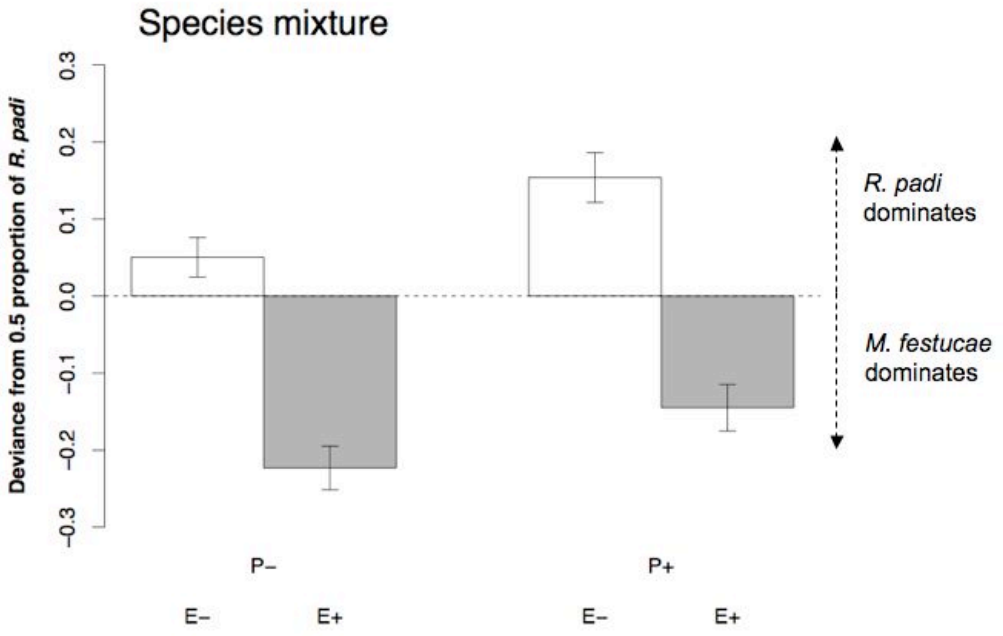

FIGURE 4. Relative abundances. Deviance of equal population densities of two aphid species in dependence of endophyte infection (endophyte-free: E -, endophyte-infected: E +) and parasitoid presence (absent: P-, present: P+). The bars show the deviance from equality of *R. padi* proportions (± SE). A positive deviance indicates a higher proportion of *R. padi* whereas a negative deviance indicates a higher proportion of *M. festucae*. The calculations were based on numbers summed over the whole 12 weeks. Endophyte ($F_{1,36}$ = 90.76, $P < 0.001$) and parasitoid ($F_{1,36}$ = 9.58, $P = 0.004$) had a significant effect, whereas the endophyte x parasitoid interaction was not significant ($F_{1,36}$ = 0.13, P = 0.722).

CHAPTER 9

NATURAL ENEMIES ACT FASTER THAN ENDOPHYTIC FUNGI IN POPULATION CONTROL OF CEREAL APHIDS

SIMONE A. HÄRRI, JOCHEN KRAUSS & CHRISTINE B. MÜLLER

ABSTRACT

1. Fast growing populations of phytophagous insects can be limited by the presence of natural enemies and by alkaloids that are produced by symbiotic associations of many temperate grass species with endophytic fungi. It is unclear if and how acquired plant defences derived from endophytic fungi interact with natural enemies to affect phytophagous insect populations.

2. To assess the relative importance of endophytic fungi compared to that of natural enemies on the population dynamics of phytophagous insects, we carried out a fully factorial field experiment, in which the presence of natural enemies and the presence of endophytic fungi were manipulated simultaneously. Target colonies of aphids were monitored for eight weeks starting from their natural appearance in the field to the end of the aphid season.

3. We show that on *Lolium perenne* increased natural enemy densities reduced the individual numbers of two common cereal aphids, *Rhopalosiphum padi* and *Metopolophium festucae*.

4. The presence of the endophytic fungi *Neotyphodium lolii* reduced the number of *M. festucae* but did not affect the number of *R. padi*. The reduction in *R. padi* numbers by predators and parasitoids was not influenced by the presence of endophytes. For adult *M. festucae* however, the negative effects of natural enemies were significant only in the absence of endophytes.

5. Over the duration of the experiment, the effect of natural enemies on aphid colony growth was much stronger than the effect of the endophytic fungi *N. lolii*, presumably because predator and parasitoid action on aphid colonies is much faster than any effects of endophytes.

6. Our results demonstrate that with simultaneous action of decreased plant quality through acquired endosymbionts and of natural enemies, aphid populations are controlled more strongly by natural enemies. The plant - endosymbiont association may have effects on whole insect food webs but the effect was not detectable in the number of

phytophagous insects in the field.

INTRODUCTION

The control and limitation of natural herbivore populations are central themes of ecological research and studies have suggested that a variety of biotic and abiotic factors control herbivore populations. Hairston, Smith and Slobodkin (1960) formulated the simple hypothesis that the world is green because natural enemies control and limit herbivores, which prevents them from totally depleting green plants (HSS-hypothesis). Subsequently, it has been repeatedly shown that natural enemies can limit herbivore abundance (Schmitz, Hambäck & Beckerman 2000; Terborgh *et al.* 2001) and that such effects of natural enemies on herbivores can result in increased abundance and productivity of primary producers (Pace *et al.* 1999). Some herbivorous pest insects, such as aphids, show particularly strong population declines in the presence of natural enemies (Müller & Godfray 1999; Schmidt *et al.* 2003; Müller, Fellowes & Godfray 2005), a fact that represents the basis for biological pest control (Beddington, Free & Lawton 1978; Fox *et al.* 2005). Alternative models suggest that herbivores are mainly donor-controlled and limited by low food plant abundance (Ohgushi & Sawada 1985) or resource quality (Hunter & Price 1992; Price 2002). The quality of the resource may be altered by the plants themselves through e.g. the production of secondary plant metabolites (Murdoch 1966; Polis & Strong 1996). The importance of natural enemies compared to the importance of resources on herbivore population growth has been the subject of heated debates but there is consensus now that both forces may interplay in herbivore population control (Leibold 1996; Denno *et al.* 2003; Sinclair, Mduma & Brashares 2003). There are a diverse range of theoretically possible interactions between resources (e.g. plant resistance against herbivores) and natural enemies (e.g. biological control; Hare 1992; Bottrell, Barbosa & Gould 1998). For example, in an additive relationship the effects of host plant resistance and natural enemies on herbivore numbers are independent and the total impact on herbivore abundance can be predicted based on each effect separately. In contrast, host plant resistance and natural enemies may interact synergistically, whereby the effects of natural enemies on herbivore abundance may be greater at higher levels of plant resistance. Alternatively, in an antagonistic interaction model, host plant resistance may affect natural enemies more than herbivores and thus, the plant itself disrupts the biological control by natural enemies (Hare 1992; Hare 2002). These different interactions of resources and natural enemies on herbivore population dynamics may be

important in maintaining the variability, complexity and diversity of food web interactions (Hassell *et al.* 1998; Oksanen & Oksanen 2000; Denno *et al.* 2002).

Hunter and Price (1992) and Polis and Strong (1996) have suggested that besides controlling mechanisms by resources and natural enemies, there could be a role for microbes in controlling herbivore populations and that these microbes might have been overlooked, despite the recent advances made in studies of soil food webs (Wardle 2002). In particular, microbes that live in symbiosis with plants will influence primary productivity by producing or capturing limiting resources and by producing toxic substances, all of which can affect the plant's quality for herbivore consumers (Omacini *et al.* 2001; Finkes *et al.* 2006). Symbiotic microbes, such as mycorrhiza (van der Heijden *et al.* 1998) and rhizobia (Vitousek *et al.* 1987), have been shown to influence the community structure of plants, which could affect herbivore populations. Fungal endophytes of plants are less well studied, but may have equally strong effects on consumer food webs (Omacini *et al.* 2001), plant community composition (Clay, Holah & Rudgers 2005; Rudgers *et al.* 2007), or even ecosystem functioning (Rudgers, Koslow & Clay 2004). Thus, these plant-associated microbes can mediate the interactions between plants, herbivores and natural enemies. The effect of endophytes on herbivores has been classified as direct, because the fungus produces the herbivore toxic compounds itself (Hemken & Bush 1989). The effects of endophytes on natural enemies are either direct (natural enemies feeding upon toxins accumulated in their host tissues) or indirect (density-mediated by a reduction in herbivore abundance or trait-mediated by e.g. reduced developmental time of the herbivores; Abrams 1995; Faeth & Bultman 2002).

Fungal endophytes are commonly associated with many different species of plants. Endophytes of the genus *Neotyphodium* (Ascomycota; Clavicipitaceae) are endosymbionts that complete their whole life cycle within the tissue of cool-season grasses (Clay & Schardl 2002). These *Neotyphodium* types live sheltered between the cells of the host plant and propagate vertically via the host plant's seeds only (Clay 1990). The grass - fungus association produces a variety of alkaloids, of which for *Lolium perenne* Linnaeus the most important are peramine, lolitrem B and ergot alkaloids (Schardl, Leuchtmann & Spiering 2004). Peramine is a potent insecticide, whereas ergot alkaloids and lolitrem B are toxic to grazing mammals (Bush, Wilkinson & Schardl 1997; Schardl *et al.* 2004). The exact composition and concentration of alkaloids in the grass depends on the plant host species, the genotype of the grass and the endophyte strain (Bush *et al.* 1997; Hunt & Newman 2005).

Numerous studies have tested whether endophyte infection alters the resistance of grasses to herbivory (Eichenseer & Dahlman 1992; Tibbets & Faeth 1999; Wilkinson *et al.* 2000; Brem & Leuchtmann 2001; Bultman & Bell 2003; Richmond *et al.* 2004; Hunt & Newman 2005; Meister *et al.* 2006). Plant resistance to at least 23 species of insects in 10 families and 5 orders has been described for fungal endophytes associated with agronomically important perennial ryegrass, *L. perenne* and tall fescue, *L. arundinacea* Schreber (Breen 1994). The aphid *Rhopalosiphum padi* Linnaeus shows poor reproduction and survival on endophyte-infected *L. arundinacea* (Eichenseer & Dahlman 1992; Bultman & Bell 2003), reduced population densities on endophyte-infected *L. multiflorum* Lamarck (Omacini *et al.* 2001) and reduced population densities on endophyte-infected *L. perenne* that derives from reduced longevity and fecundity on such infected grasses (Meister *et al.* 2006). Only a few studies have looked at the potential for endophyte effects to be transmitted to higher trophic levels (Müller & Krauss 2005). However, there could be direct or indirect effects of certain endophytic fungi on parasitoids (Bultman, McNeill & Goldson 2003; S. A. Härri, unpublished data) and predators (de Sassi, Müller & Krauss 2006). Parasitoids as well as ladybird predators show measurable disadvantages when constrained to consume aphids on endophyte-infected food plants in the laboratory. Without alternative prey on uninfected plants these natural enemies would lose their effectiveness in controlling aphid populations in the long term which may result in higher herbivory for plants by insects that are not sensitive to the endophyte toxins. In the field, the parasitoid community based on endophyte-infected *L. multiflorum* has fewer species; fewer interactions with aphids and the strength of these interactions are more evenly distributed within the food web than on uninfected grasses (Omacini *et al.* 2001).

Endophytic fungi and natural enemies are two factors that could limit the abundance of aphids in the field but so far no study has investigated the relative importance of natural enemies compared to endophytic fungi and the subsequent change in plant quality on aphid population growth. By simultaneously manipulating the presence of both natural enemies and endophytes, we tested for the relative strength of the effects of natural enemies and endophytes, and the nature of their interaction on aphid numbers in the field. Based on previous results, we hypothesised that the presence of endophytes would have a strong negative effect on *R. padi* numbers, and that the reduced densities and lower quality of the prey would subsequently be less attractive to natural enemies freely moving in the field. Therefore, we expected to see strong negative effects on aphid

numbers by natural enemies only on uninfected plants as suggested by the antagonistic interaction model (Hare 1992; Hare 2002).

MATERIALS AND METHODS

Material

Our model system consisted of the endophytic fungi *Neotyphodium lolii* Glenn, Bacon and Hanlin, a specialist on the perennial ryegrass *L. perenne*, and the two grass aphid species *R. padi* and *Metopolophium festucae* Theobald.

The *L. perenne* cultivar used in our experiment was Samson (*L. perenne*, Grassland Samson), which was either endophyte-free (E -: identity number A 11104) or endophyte-infected (E +: identity number A 12038). The infection was lost by specifically selecting seeds where the endophyte transmission was unsuccessful (B. Tapper, personal communication). The infection status was checked microscopically by staining 30 seeds (Saha, Jackson & Johnson-Cicalese 1988) of each, the infected (93 % infection) and the uninfected (0 % infection) seeds. The endophyte *N. lolii* produces the alkaloids Lolitrem B and Peramine, which both were confirmed for the infected plant cultivar (Lolitrem B (0.4 ! g) and Peramine (7.5 ± 1.5 ! g/g); Krauss *et al.* 2007a)

The laboratory culture of *R. padi* was started with few individuals collected near the University of Zürich, Switzerland in May 2003. The culture was kept in a controlled-environment room at 20ºC and 16:8 hrs light: dark cycle on *L. perenne* ARION (commercially available endophyte-free fodder grass (staining of 30 seeds: 0% infection), provided by FAL Reckenholz, Switzerland). We assumed that the laboratory culture consisted of one or a few *R. padi* clones only.

Experimental design

To simultaneously test for the effects of natural enemies and endophyte infection on aphid population density in the field, a 3 x 2 full factorial design with three levels of 'predation' and two levels of endophyte infection replicated within ten randomised blocks was set up, resulting in a total of 60 experimental pots. The factor 'infection' consisted of endophyte-free *L. perenne* (E -) and endophyte-infected *L. perenne* (E +). Thirty pots (Ø 30 cm) were sown on 11 February 2004 with E + seeds and 30 pots with E - seeds (0.35 g of seeds per pot). The pots were left in the greenhouse for three months and the grass was cut at a height of 20 cm at the end of this growing period. Afterwards, the pots were transferred to the field and left to acclimatise to outdoor conditions for four weeks. Within

each block, the pots were placed at an interval of 1 m. The grass was cut again to 15 cm height after seven days to facilitate insect counts. All pots were always covered with nylon mesh to prevent natural aphid colonisation before the start of the experiment on 7 June 2004. In the surrounding fields, aphids on grasses were first observed shortly after the start of our experiment.

The factor 'predation' consisted of ***1)*** 'predator exclusion', ***2)*** 'predator presence' and ***3)*** 'cage control'. The 'predation' treatments were achieved with the following methods: ***1)*** Predator exclusion: a round wire cage (mesh size 0.6 cm) with a diameter of 32 cm and a height of 80 cm was coated with insect glue (Tangle-Trap ®, Andermatt Biocontrol AG, Grossdietwil, Switzerland) and fitted over the pots to ensure that predators and parasitoids were intercepted as effectively as possible when attempting to enter the cage, ***2)*** predator presence: the pots had no cage, ***3)*** cage control: to control for the effect of the cage on the microclimate under the cage, an uncoated control cage was used. This control cage was constructed the same way as for treatment ***1)***, but had four openings (4 cm x 40 cm) and no coating of insect glue.

Two weeks before the pots in the field were stocked with aphids, for each of the sixty pots, ten adult *R. padi* were randomly selected from the base culture. They were placed in a Petri dish together with wet filter paper and a piece of *L. perenne* ARION, where they were left to reproduce to obtain a similar mix of aphid life stages. For each Petri dish the filter paper was changed and new cuttings of *L. perenne* ARION were added every second day. After 14 days, the aphids in each Petri dish were counted and randomly assigned to the experimental pots. The starting density of *R. padi* did not differ between the treatments (linear mixed effects model with block as random factor; ln [x + 1] - transformed: $F_{(\text{predation})\ 2,45} = 1.15$, $P = 0.325$, $F_{(\text{infection})\ 1,45} = 0.79$, $P = 0.378$, $F_{(\text{predation x infection})\ 2,45} = 0.06$, $P = 0.946$). However, it is possible that additional *R. padi* may have colonised the experimental pots naturally, as was the case for *M. festucae*.

One week after the start of the experiment on 7 June 2004, the number of aphids and the number of predators and mummies (aphids attacked by parasitoids) were counted by searching each pot for five minutes. Aphid mummies and all predators on the predator exclusion pots were removed. These counts were repeated every week for eight weeks, after which time the experiment was terminated as no aphids (or only a very small number) were observed on the experimental pots and on grasses in the surrounding grasslands. Therefore, the experiment covered the whole natural cereal aphid season. At the end of the experiment on 23 August 2004, the aboveground plant biomass was

harvested and dried for three days at 80 °C and then weighed.

Microclimatic differences

To assess possible microclimatic differences among treatments caused by the cages, temperature (Alma-digit ad 15 th Precision meter, Amarell Electronic, Kreuzwertheim, Germany) and humidity (EXOTERRA hygrometer, Zum Goldfisch Fiwe-Aquarium GmbH, Basel, Switzerland) were measured on a cloudy day (5 August 2004) and on a sunny day (17 August 2004). In each block, the temperature and humidity at the centre of a pot covered with a glue-coated cage ('predator exclusion'), a pot covered with a control cage ('cage control') and a pot without cage ('predator presence') were measured three times during one day: in the morning (08:00 - 09:00), at noon (12:00 - 13:00) and in the evening (17:00 - 18:00). In each case two pots from different 'predation' treatments were measured simultaneously. A three-way ANOVA with 'predation', 'day', and 'daytime' modeled as factors was used to analyse the absolute differences between each treatment combination. There were no significant differences in temperature ($F_{2,72} = 0.67$, $P = 0.516$) or humidity ($F_{2,72} = 0.94$, $P = 0.396$) between the three 'predation' treatments.

Statistical analyses

All statistical analyses were performed in R (version 2.0.1 for MacOS X). Means were expressed as grand means: the mean for each treatment combination averaged over all eight sampling weeks. The grand means were calculated for the number of aphids (ln [x + 1]-transformed), mummies, predators and plant biomass. The temporal dynamics over the eight sampling weeks for the number of aphids were also analysed. However, as there were no differences in the temporal pattern among the treatments, and the qualitative results were the same as for the grand means, we only present the results for the grand means here. Grand means were analysed using a linear mixed effects model (lme-function) with 'predation' treatment and 'infection' treatment as factors and block as a random effect. For all linear mixed effects models the maximum likelihood method was used for model fit and the general positive-definite symmetric variance-covariance structure for the random effects (Pinheiro & Bates 2000). For each model, independence and normal distribution of within-group errors and normal distribution of random effects were assessed and transformations were applied where required.

The proportion of alates (winged individuals) within one colony was very low (grand means range between 1.27 % and 2.36 % for *R. padi* and between 1.58 % and

13.74 % for *M. festucae*). The proportions of alates for *R. padi* were analysed using a linear mixed effects model as residuals met the assumption of normal distribution and homoscedasticity whereas the proportions of alates for *M. festucae* were analysed with a generalised linear mixed model with binomial error distribution (glmmPQL-function), as residuals did not meet the above-mentioned assumptions. The proportion of alates for both species did not differ among the treatments (*R. padi*: $F_{(\text{predation})\ 2,45} = 2.35$, $P = 0.107$, $F_{(\text{infection})\ 1,45} = 0.09$, $P = 0.765$; *M. festucae*: $F_{(\text{predation})\ 2,36} = 0.59$, $P = 0.559$, $F_{(\text{infection})1,36} < 0.001$, $P = 0.990$). Overall, the results did not differ qualitatively for the separate analyses of number of adults, nymphs or the total number of aphids for *R. padi*. Therefore, the results presented are total number of aphids (number of adults + number of nymphs + number of alates). For *M. festucae*, the results did not differ qualitatively for the separate analyses of number of nymphs and total number of aphids, however the results differed for the separate analysis on adult *M. festucae*. Thus, results are shown separately for adult *M. festucae* and total number of *M. festucae* (number of adults + number of nymphs + number of alates).

RESULTS

The presence of natural enemies reduced *R. padi* numbers whereas the presence of the endophytic fungi had no significant effect on *R. padi* numbers (Table 1; Fig. 1a). The interaction between the presence of endophytes and presence of natural enemies was not significant (Table 1; Fig. 1a).

Metopolophium festucae colonies were negatively affected by the presence of natural enemies and the endophyte infection of *L. perenne* (Table 1). For the total number of *M. festucae*, the interaction between endophyte presence and presence of natural enemies was not significant (Table 1). However, for adult *M. festucae*, the interaction was marginally significant with presence of endophytes decreasing the number of adult *M. festucae* only in the 'predator exclusion' treatment (Table 1, Fig. 1b).

Plant biomass at the end of the experiment was not affected by either the predator treatment ($F_{2,45} = 0.34$, $P = 0.715$), or by endophyte presence ($F_{1,45} = 0.006$, $P = 0.940$), nor by the interaction between the two treatments ($F_{2,45} = 0.25$, $P = 0.780$).

The number of natural enemies observed during the experiment was generally very low (Table 2). The numbers of generalist (Nabidae, Miridae, and Araneae) and specialist (Coccinellidae, Chrysopidae, and Syrphidae) aphid predators that were collected on the 'predator exclusion' treatment were lower ($F_{2,45} = 3.82$, $P = 0.029$) and

number of parasitoid mummies tended to be fewer on the 'predator exclusion' treatment ($F_{2,45} = 2.68$, $P = 0.079$) than on the 'predator presence' and on the 'cage control' treatments. Both predators and parasitoids (mummies) did not differ in their abundances on endophyte-infected and endophyte-free *L. perenne* (predators: $F_{1,45} = 0.22$, $P = 0.645$; mummies: $F_{1\,45} < 0.001$, $P = 0.994$).

DISCUSSION

Our experiment demonstrated that the access of natural enemies to aphid colonies in the field significantly decreased aphid numbers, whereas the presence of the endophytic fungi *N. lolii* only reduced the size of *M. festucae* colonies but not that of *R. padi*. Except for adult *M. festucae*, we did not observe the expected antagonistic interaction between endophyte presence and natural enemy presence. For *M. festucae* adults, the negative effects of natural enemies were significant only in the absence of endophytes. Thus, endophyte infection and presence of natural enemies interacted in an antagonistic way as expected. This might be explained by a reduced predator activity on endophyte-infected *L. perenne*. In a laboratory experiment, primary parasitoids were less fecund when developing in *M. festucae* from endophyte-infected plants (S. A. Härri, unpublished data). We therefore expected to find fewer parasitoid mummies and predators on endophyte-infected *L. perenne*. This expectation was not supported by our data. However, arthropod predators were particularly difficult to quantify accurately with our set-up, as they are highly mobile. Overall, the effect of the endophyte on aphid numbers was weaker than the reduction in aphid numbers caused by the presence of natural enemies. For both aphid species the strong negative effects of predators on aphid numbers appeared to override any influence by the *N. lolii* infection of *L. perenne*.

An explanation for stronger natural enemy effects in comparison to endophyte effects over the duration of the experiment may be the difference in the timescales over which natural enemies and endophytes act. In comparison to aphid natural enemies, endophytes act slower by negatively influencing the life history of individual aphids through reduced longevity and fecundity (Meister *et al.* 2006), which results in a delayed decrease in aphid number. In the laboratory, a decrease in *R. padi* colony size on endophyte-infected *L. perenne* is only visible after more than a week (Meister *et al.* 2006). In contrast, arthropod predators and parasitoids are mobile and move quickly from plant to plant, reducing or erasing entire aphid colonies. Strong predator impacts on aphid colonies have been demonstrated in natural (Müller & Godfray 1999; Weisser 2000;

Müller *et al.* 2005) and agricultural systems (Schmidt *et al.* 2003; Fox *et al.* 2005). Parasitoids were probably the most efficient natural enemies in our experiment. These natural enemies have been shown to cause up to 100% parasitism rate in natural field systems (Weisser 2000) and in agricultural cereal systems, where parasitoids are the most efficient natural enemies at reducing aphid densities (Schmidt *et al.* 2003). Apart from parasitoids, the most abundant specialist predators in our experiment were ladybirds that act as voracious aphid consumers in both their larval and adult life stages (Obrycki & Kring 1998). Specialist predators of aphids are indeed successfully used in pest control of cereal aphids (Cardinale *et al.* 2003).

In a field study on *L. multiflorum*, endophytes had strong effects on *R. padi* that drove most changes in abundance and richness of higher trophic parasitoid levels (Omacini *et al.* 2001). Similarly, in laboratory experiments, effects of endophytes on *R. padi* are strong (Meister *et al.* 2006) and are transmitted up the food chain, affecting life-history traits of predators (de Sassi *et al.* 2006). Based on these results, we expected endophytes to have a strong effect on *R. padi* numbers, which in turn would decrease the number of predators, and parasitoids on endophyte-infected plants. However, this was not observed in our experiment; *R. padi* populations were not significantly affected by endophytes and the number of natural enemies did not differ between the two endophyte treatments. The weak effect of *N. lolii* on *R. padi* numbers is puzzling because the presence of peramine would be enough to negatively affect this species (Tanaka *et al.* 2005), despite the absence of insect-toxic lolines that are not produced by *N. lolii* but are known to have a stronger impact on herbivores (Hunt & Newman 2005). This discrepancy between the negative effects of endophytes on aphids and natural enemies observed in the laboratory compared to field studies was described before (Krauss *et al.* 2007a; Krauss *et al.* 2007b). There are several possible reasons why this may occur; one being the relatively low peramine concentration in our experimental plants. Additionally, different abiotic and biotic factors are known to influence alkaloid concentration of endophyte-infected plants in the field (Bultman & Bell 2003; Cheplick 2004; Lehtonen, Helander & Saikkonen 2005; Salminen *et al.* 2005; Meister *et al.* 2006). It is also possible that there are differences in the clonal composition of *R. padi* on infected and uninfected plants. Certain clones of *R. padi* may perform better on infected plants and could be selected for over the course of the season (A. Bieri, unpublished). Although, we controlled these variables by keeping the same conditions across all treatments and stocking aphids from a laboratory culture, some of them may be responsible for the different outcome of our field

experiment compared to laboratory experiments.

The other aphid species present in our experiment, *M. festucae,* showed lower numbers on *L. perenne* infected with *N. lolii* and lower numbers when natural enemies were present. The reduction in abundance caused by endophytes was mainly expressed in the absence of predators, especially for adult *M. festucae*. In all previous studies, *M. festucae* was never affected as strongly as *R. padi* by the presence of endophytes (Omacini *et al.* 2001; S. A. Härri, unpublished data). In our experiment *M. festucae* was not stocked in a controlled way but colonised our experimental pots naturally. Therefore, it is possible that these colonisers were discriminating against infected grasses and colonised mainly uninfected plants or *M. festucae* was indeed growing less well on infected plants. We are unable to differentiate between the two possibilities with this experiment, and more detailed preference tests are needed.

Other studies showed strong predator control, especially in aquatic systems, where the removal of top predators leads to increases in herbivores and reductions of primary producers (Carpenter, Kitchell & Hodgson 1985; Wootton & Power 1993; Estes *et al.* 1998), but many convincing examples from terrestrial systems exist as well (Marquis & Whelan 1994; Terborgh *et al.* 2001). Such cascading negative effects on the biomass of primary producers by removal of predators were not observed in our experiment, presumably because feeding by sap-sucking aphids does not remove large proportions of plant tissue (but see Müller *et al.* 2005), or because the control of the aphids by predators, and to a certain degree by endophytes, was strong enough to keep aphid numbers moderately low. The strong effect of natural enemies on aphid numbers compared to the lack of an effect of aphids on grasses suggests strong direct effects between adjacent trophic levels but no cascading, indirect effects on plant biomass. Food web theory predicts that systems on plants that have effective chemical or mechanical defences against herbivores should show strong effects of natural enemies on herbivores but weak indirect effects of natural enemies for primary plant producers (Polis & Strong 1996; Schmitz *et al.* 2000).

To our knowledge, this is the first field study incorporating microorganisms in an experimental analysis of the relative importance of natural enemies *vs*. acquired plant defence through fungal plant endosymbionts. The simultaneous manipulation of the endophytic symbiont in the food plant and of the presence of natural enemies allowed us to show that aphid numbers were strongly limited by natural enemies but at the same time only weakly affected by the presence of endosymbionts. The exception was the adult *M.*

festucae population, for which both forces interacted and negative effects of natural enemies were only present when endophytes were absent. We also discovered some inconsistency with other experimental results on the same system in the laboratory, which suggests that the crucial interactions determining aphid population growth may be more complex than those observed in simple laboratory communities and may be dependent on exact field conditions. Despite finding a strong predator effect on aphid numbers in our experiment, the complexity and potential changes of interactions within whole insect food webs that may be caused by microbial endosymbionts remains an open field for research.

ACKNOWLEDGEMENTS

We thank Susanne Müller, Julia Nüscheler, Tobias Züst and Claudio de Sassi for the help in the field. Jonathan Newman, Ulrich Steiner, Dennis Hansen, Tom Jones and two anonymous reviewers for helpful comments on the manuscript. Brian Tapper (AgResearch, New Zealand) provided the *L. perenne* seeds. The study was financially supported by the Swiss National Science Foundation (Grant number 631-065950 to cbm).

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TABLES

TABLE 1. The results of the linear mixed effect models showing the effect of predator and parasitoid presence manipulation ('predation'), the presence of endophytes ('infection') and the interaction between 'predation' and 'infection' on the number of *R. padi* (adults, nymphs and alates pooled), on the number of *M. festucae* (adults, nymphs and alates pooled) and on the number of adult *M. festucae*. For *R. padi*, the separate analyses on the number of adults and the number of nymphs reveal a similar pattern as for total number and results are not shown. The number of *M. festucae* nymphs show a similar pattern as the total number of *M. festucae* and thus the result is not shown. Data were ln [x+1]-transformed for the analyses.

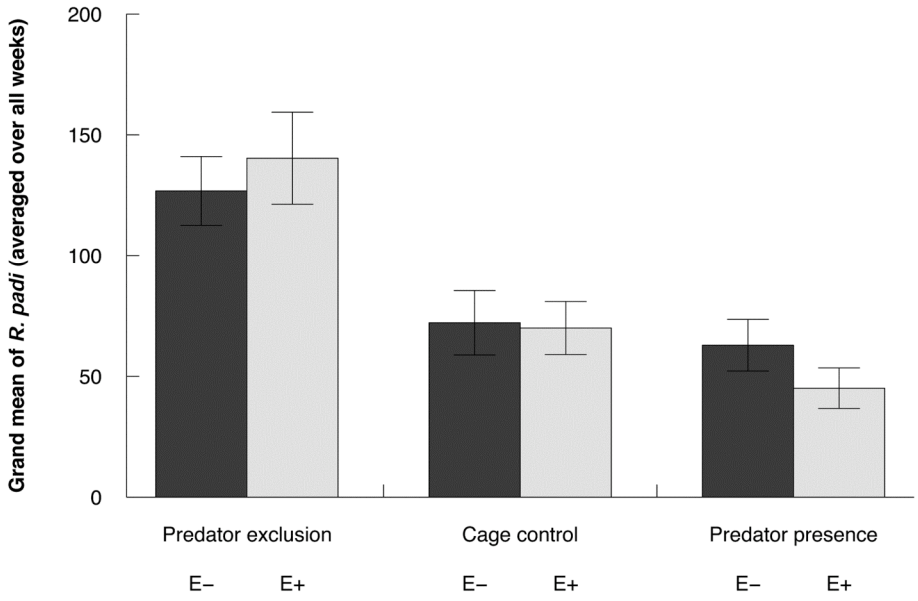
Source of variation	*R. padi*	*M. festucae*	Adult *M. festucae*
Infection	$F_{1,45} = 0.30$ $P = 0.585$	$F_{1,45} = 7.49$ $P = 0.010$	$F_{1,45} = 7.81$ $P = 0.0076$
Predation	$F_{2,45} = 17.29$ $P < 0.0001$	$F_{2,45} = 16.23$ $P < 0.0001$	$F_{2,45} = 11.12$ $P = 0.0001$
Infection x Predation	$F_{2,45} = 0.77$ $P = 0.469$	$F_{2,45} = 1.12$ $P = 0.335$	$F_{2,45} = 3.16$ $P = 0.0518$

TABLE 2. The mean number (± SE) of natural enemies averaged over all eight sampling weeks for the 3 x 2 treatment combinations. The number in brackets shows the total number of natural enemies found during the course of the experiment. In the 'predator exclusion' treatment, all predators were manually removed weekly, whereas in the other treatments predators were left on the pots. The 'predator exclusion' treatment did not totally prevent predators from colonising the experimental plants but it reduced the number of individuals for most groups of natural enemies. In addition, the manual removal of natural enemies in the 'predator exclusion' treatment should have reduced predator pressure on aphid colonies.

Predation treatment	Endophyte treatment	Coccinellidae (adults + larvae)	Chrysopidae (larvae)	Syrphidae (larvae)	Nabidae and Miridae	Araneae	Parasitoid mummies	Total
Predator exclusion	E -	0.18 ± 0.05 (14)	0 ± 0 (0)	0.08 ± 0.04 (6)	0.06 ± 0.04 (5)	0.10 ± 0.04 (8)	0.39 ± 0.14(31)	64
	E +	0.21 ± 0.05 (17)	0 ± 0 (0)	0.05 ± 0.03 (4)	0.08 ± 0.03 (6)	0.10 ± 0.04 (8)	0.66 ± 0.17 (53)	88
Predator presence	E -	0.18 ± 0.03 (14)	0.13 ± 0.06 (10)	0.04 ± 0.03 (3)	0.04 ± 0.02 (3)	0.15 ± 0.06 (12)	1.25 ± 0.34 (100)	142
	E +	0.26 ± 0.07 (21)	0.04 ± 0.03 (3)	0.05 ± 0.03 (4)	0.10 ± 0.05 (8)	0.30 ± 0.07 (24)	0.89 ± 0.32 (71)	131
Cage control	E -	0.21 ± 0.09 (17)	0.03 ± 0.02 (2)	0.03 ± 0.02 (2)	0.25 ± 0.09 (20)	0.26 ± 0.07 (21)	0.80 ± 0.29 (64)	126
	E +	0.19 ± 0.07 (15)	0 ± 0 (0)	0.08 ± 0.04 (6)	0.19 ± 0.06 (15)	0.35 ± 0.11 (28)	0.79 ± 0.25 (63)	127

FIGURES

a)


Grand mean of R. padi (averaged over all weeks)
200
150
100
50
0
Predator exclusion
Cage control
Predator presence
E–
E+
E–
E+
E–
E+

b)

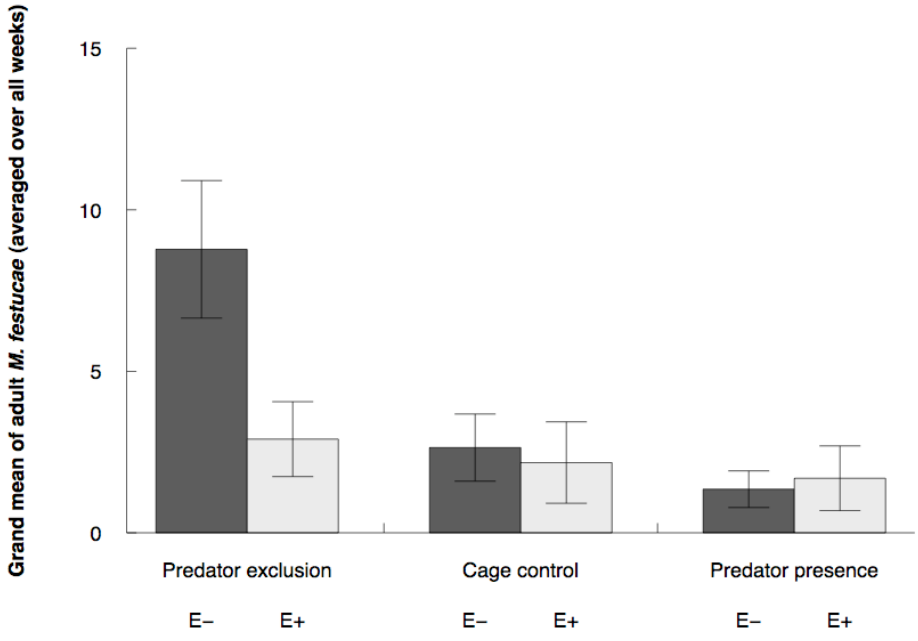

Grand mean of adult M. festucae (averaged over all weeks)
15
10
5
0
Predator exclusion
Cage control
Predator presence
E–
E+
E–
E+
E–
E+

FIGURE 1. The grand mean (± SE) of a) *R. padi* and b) adult *M. festucae* (both averaged over all eight sampling weeks) for the 3 x 2 treatment combinations. The presence of predators and parasitoids reduced the numbers of *R. padi* and of *M. festucae* whereas the endophyte infection had no effect on the number of *R. padi* but decreased the number of *M. festucae* (Table 1). The interaction between 'predation' treatment and endophyte infection was not significant for *R. padi* but for number of adult *M. festucae* (Table 1). Light grey bars show aphid numbers on endophyte infected (E+) plants and dark grey bars aphid numbers on endophyte-free plants (E-).

CHAPTER 10

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science."

ALBERT EINSTEIN

Functional Ecology 2007
21, 107–116

Effects of fertilizer, fungal endophytes and plant cultivar on the performance of insect herbivores and their natural enemies

JOCHEN KRAUSS*, SIMONE A. HÄRRI*, LOWELL BUSH‡, RENÉ HUSI*, LAURENT BIGLER§, SALLY A. POWER¶ and CHRISTINE B. MÜLLER*

**Institute of Environmental Sciences, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, ‡Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546-0091, USA, §Institute of Organic Chemistry, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, ¶Division of Biology, Imperial College London, Silwood Park Campus, Ascot, Berkshire SL5 7PY, UK*

Summary

1. Endophytic fungi are associates of most species of plants and may modify insect community structures through the production of toxic alkaloids. Fertilization is known to increase food plant quality for herbivores, but it is also conceivable that additional nitrogen could increase the production of the insect toxic alkaloid, peramine, in endophyte-infected plants.
2. The relative importance of soil fertility and endophyte infection on herbivores and their natural enemies is unknown. As performance of the host plant is often affected by an interaction between endophyte infection and genetic background, four different plant cultivars were tested. The main questions addressed in this study were whether plant cultivar and fertilizer addition to endophyte-infected and endophyte-free *Lolium perenne* affect alkaloid concentrations, plant life-history traits and the abundances of aphid species and their parasitoids.
3. In a full factorial outdoor experiment we found a strong positive effect of fertilizer on plant biomass and on the abundance of aphids and parasitoids. While plant traits differed between cultivars, there was little effect of cultivar on either aphid or parasitoid abundance. Only endophyte-infected plants contained alkaloids, and the concentration of peramine was enhanced in fertilized plants. However, endophyte infection had no negative effect on aphid or parasitoid abundances. Plant traits were only weakly influenced by endophyte infection in the field, which contrasts with plant growth room studies, where both germination rate and plant height were influenced by endophyte–cultivar interactions.
4. The generally weak effects of endophytes in the outdoor experiment could be explained by various additional constraints under field conditions and the relatively low peramine concentration that we observed.

Key-words: cereal aphids, endophytic fungi, *Neotyphodium lolii*, parasitoids, trophic cascades.

Functional Ecology (2007) 21, 107–116
doi: 10.1111/j.1365-2435.2006.01216.x

Introduction

Most plants have microbial associates (Clay 2004) and such associations may alter processes of plant succession (Clay & Holah 1999), general relationships between plant species diversity and productivity (Rudgers, Koslow & Clay 2004), and insect food web interactions (Omacini *et al.* 2001). Endophytic fungi of cool season grasses are often seen as mutualistic symbionts, with the fungi receiving shelter, nutrients and transmission to the next generation via grass seeds, and host grasses having higher stress tolerance and herbivore resistance (Schardl, Leuchtmann & Spiering 2004; Müller & Krauss 2005). Cereal aphids, which are common grass herbivores, often show strong negative responses when feeding on endophyte-infected agronomic grass species (Breen 1994; Hunt & Newman 2005; Meister *et al.* 2006). The grass–fungus association produces a cocktail of alkaloids and, in the grass *Lolium perenne*, the main insect toxic substance is peramine. The alkaloids

†Author to whom correspondence should be addressed.
E-mail: jkrauss@uwinst.unizh.ch

ergovaline and lolitrem B are also found in *L. perenne* (Spiering *et al.* 2002; Schardl *et al.* 2004), the latter being responsible for ryegrass staggers in sheep (Schardl *et al.* 2004). All alkaloids vary in concentration and distribution within a single host plant (Fannin, Bush & Siegel 1990; Ball, Prestidge & Sprosen 1995; Keogh, Tapper & Fletcher 1996; Ball *et al.* 1997; Spiering *et al.* 2002) and toxic effects depend on environmental conditions (Faeth, Bush & Sullivan 2002) and the genetic backgrounds of fungus and grass host (Roylance, Hill & Agee 1994; Faeth *et al.* 2002). As nitrogen is a key component of alkaloids, it could be expected that nitrogen addition will increase the alkaloid concentration in infected grasses (Lyons, Plattner & Bacon 1986; Marks, Clay & Cheplick 1991; Latch 1993; Faeth & Fagan 2002). Indeed, concentrations of lolitrem B and peramine have been shown to be higher in well fertilized ryegrass compared with poorly fertilized plants (Latch 1993). However, even though plant nitrogen concentrations typically increase in response to fertilization (Davidson & Potter 1995), Faeth *et al.* (2002) found that the peramine concentration of Arizona Fescue was not altered by fertilizer treatment.

Generally, aphid densities are enhanced when plants are grown with additional fertilizer (Honek 1991; Davidson & Potter 1995); this could result in a conflicting situation for aphids on endophyte-infected plants where insect growth rates are enhanced by fertilization, but reduced through higher concentrations of toxic alkaloids. The aphid *Rhopalosiphum padi* benefits from fertilizer addition, showing higher growth rates on fertilized plants of *Lolium* (formerly *Festuca*) *arundinacea*. However, when the grass is infected with the endophyte *Neotyphodium coenophialum*, the positive effect of fertilizer is counteracted and aphid population densities decrease (Davidson & Potter 1995). In this latter study, effects on the population densities of natural enemies of aphids were not considered. It is, however, conceivable that not only herbivores, but also their natural enemies are affected by both endophyte presence and fertilizer addition, with further feedbacks on herbivore densities. Flying predators (Müller & Godfray 1999) and particularly parasitoids (Schmidt *et al.* 2003) can have strong negative effects on aphid colony growth. Several laboratory studies on endophytes have found that predators (de Sassi, Müller & Krauss 2006) and parasitoids (Barker & Addison 1996; Bultman *et al.* 1997; Bultman, McNeil & Goldson 2003) are negatively affected by the presence of endophytic fungi. However, these studies were conducted under laboratory conditions, with insects being fed on endophyte-infected food. Providing natural enemies with a choice, under field conditions, might result in less distinct fitness losses.

As with most studies on the effects of endophytes on herbivores and predators, effects on plant life-history traits are often measured only in greenhouse experiments and only during the first few months of the lifespan of grasses (e.g. Cheplick 1998, 2004; Cheplick & Cho 2003). Field conditions may alter these results, because more species, at different trophic levels, will interact in the field, potentially resulting in higher order interactions (Wootton 1994; van Veen, Morris & Godfray 2006). In addition, all endophyte-mediated effects on plant life-history, alkaloid concentration, density of herbivores and natural enemies may be influenced by the plant's genotype or cultivar (Cheplick 1998, 2004; Faeth *et al.* 2002; Cheplick & Cho 2003; Meister *et al.* 2006). Here we present data from four agronomically important cultivars of *Lolium perenne* L., with the asexually transmitted endophyte, *Neotyphodium lolii* Glenn, Bacon and Hanlin, which relies entirely on seed production of the host plant to pass to the next generation. It would be expected that such an endosymbiont would manipulate its host plant to allocate more resources to reproduction, compared with uninfected plants.

The main aim of this study was to understand the relationships between fertilizer treatment, endophyte infection and plant cultivar on plant life-history of *L. perenne* and the associated insect population densities in the field. This was achieved by a full factorial outdoor experiment, in which insects were left to colonize the plants naturally. The main predictions addressed were that: (1) endophyte infection alters plant performance, especially the allocation of resources to reproduction; (2) fertilizer addition and grass cultivar affect plant life-history traits and these may interact with endophyte infection; (3) peramine and nitrogen concentrations are enhanced after fertilizer addition; and (4) endophyte infection decreases aphid and parasitoid abundances, but grass cultivar and fertilizer treatment modify this effect.

Materials and methods

Seeds of English ryegrass, *L. perenne* (Poaceae), of four different agronomically important plant cultivars, with and without endophyte infection by the common endophyte strain *N. lolii*, were used. The plant cultivars were Imp (*Lolium* ! *boucheanum*, Grassland Impact), Nui (*L. perenne* Grassland Nui), Pac (*L. perenne* Grassland Pacific) and Sam (*L. perenne*, Grassland Samson). Each cultivar was either uninfected (E–) or infected (E+) with the fungus *N. lolii*. All uninfected grass cultivars showed no infection after diagnostic staining of seeds, whereas 67–97% of the stained seeds of infected plants had fungal hyphae, depending on cultivar (Meister *et al.* 2006).

Establishment of plant material

In December 2003, infected and endophyte-free *L. perenne* of all four cultivars were grown on commercially available garden compost in a climate controlled plant growth room, under artificial light. Each seed was sown

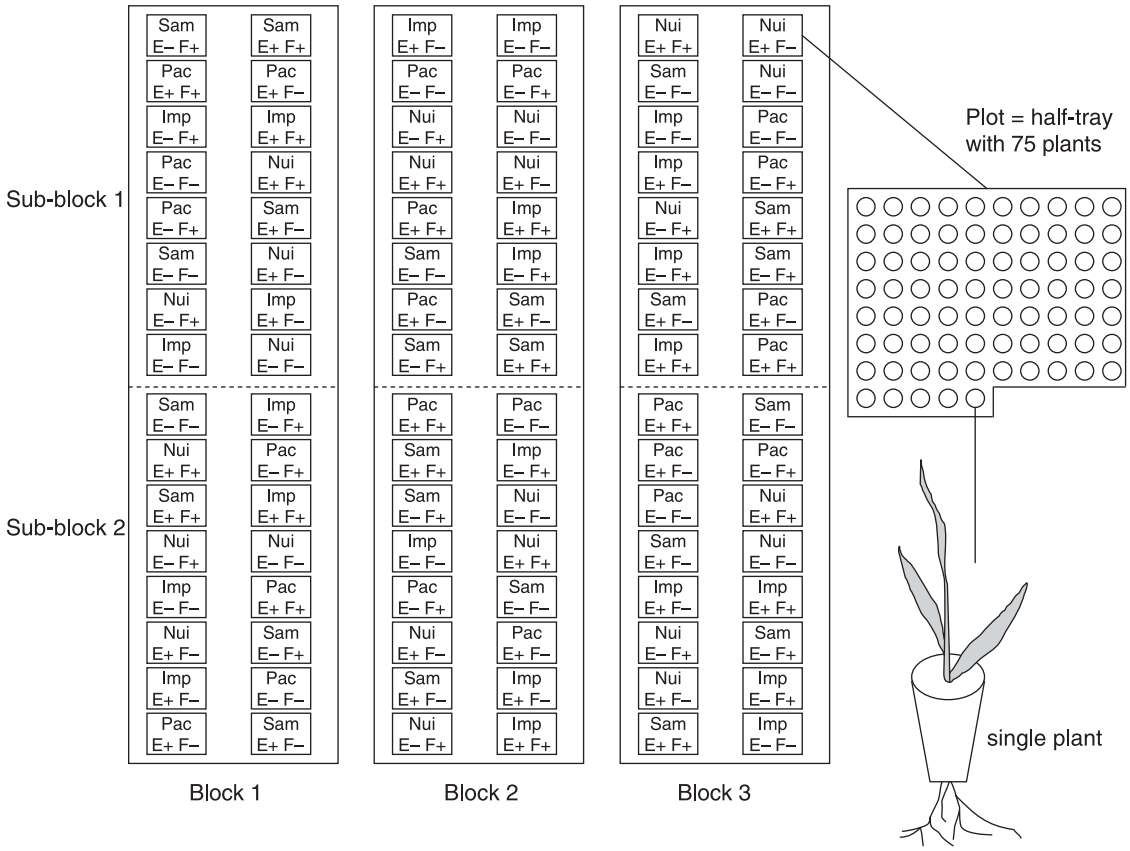


Fig. 1. The experimental design, showing the three experimental blocks with two nested sub-blocks and the within sub-blocks randomized plots (half-trays), with a total $n = 96$. Each plot contains 75 single plants in a half propagation plug tray. Imp, Nui, Pac, Sam = abbreviations for the four different grass cultivars. E–, E+ = endophyte-free and endophyte-infected plants. F–, F+ = not fertilized and fertilized plants.

separately in a cell of 48 propagation plug trays containing 150 seedling cells per tray (http://www.gvz-bolltec.ch: part number 65150). This represents 7296 single plants in total. The cells for each plant had a diameter of 2·8 cm with a volume of 17 cm^3. Each tray contained only seeds from one infection status and one cultivar, resulting in a replication of $n = 6$ trays per treatment. Thirteen days after the seeds were sown, the height of 20 randomly chosen germinated plants per tray was measured. One day later, the germination success was also assessed by counting the proportion of germinated plants per tray.

For the main experiment, plants that had not germinated after 14 days were replaced by reserve plants of the same age and the 48 propagation plug trays were moved to a greenhouse for 4 months. At the end of the 4 months all trays were split in two equally sized half-trays containing 75 plant cells (Fig. 1). Sprawling roots at the bottom were removed before planting the half-trays into the soil of three experimental blocks in a field site at the end of April 2004. The base of each experimental block was fenced to prevent mouse entry, and filled with 20 cm of normal soil from nearby agricultural lands with an estimated nitrate content of 10 mg kg^{-1} (pers. comm. Theres Zwimpfer, University of Zürich). The three blocks were placed in snail proof enclosures in an experimental field site at the University of Zürich. The experimental field site was surrounded by grassland with naturally occurring grass aphids, a nearby forest and the university buildings. The 96 half-trays (henceforth referred to as 96 plots) are considered the experimental unit, and were equally distributed between the three experimental blocks, with two nested sub-blocks (Fig. 1). Randomization of the plots took place within the sub-blocks; half of the 96 plots were fertilized with a balanced fertilizer (Wuxal–MaagAgro, N 100 g l^{-1}, P_2O_5 100 g l^{-1}, K_2O 75 g l^{-1}, B 120 mg l^{-1}, Cu 81 mg l^{-1}, Fe 190 mg l^{-1}, Mn 162 mg l^{-1}, Mo 10 mg l^{-1}, Zn 61 mg l^{-1}), at a rate equivalent to 200 kg N ha^{-1} provided in seven doses at 2-week intervals between April and July. The amount of nitrogen added was representative of typical agricultural application rates in western Europe (Carsten Thies, pers. comm., University of Göttingen, Germany). Each of the three treatments [plant cultivar (Imp, Nui, Pac, Sam), endophyte infection (E–, E+) and fertilizer (F–, F+)] were present in each sub-block once, resulting in a replication of $n = 6$ per treatment (Fig. 1). Plants were watered as required and any weeds occurring between plots were regularly removed. A minimum of approximately 20 cm between all 96 plots reduced direct competition between the plants of the different treatments. Competition within the treatment in a single plot (half-tray containing 75 plants) was reduced due to the separated cells in the half-tray. However, root competition in deeper soil may have occurred.

Plant traits and chemical analyses

In August 2004, 8 months after *L. perenne* was planted, the total number of ears per plot was determined to estimate reproductive allocation. Thereafter, the above-ground biomass of all plots was harvested at ground level, and divided into ear and shoot biomass to provide a further estimate of the allocation to reproductive vs. vegetative growth. The oven-dried biomass was weighed and the number of spikelets and length of ears were measured for 10 randomly chosen ears per plot.

Prior to harvest, five randomly chosen shoots with ears were selected from each treatment plot. These were immediately frozen in liquid nitrogen, ground in a mill and dried by lyophilization for chemical analyses. Analyses of carbon and nitrogen concentrations were carried out using a CHNS-932 determinator (LECO Corporation, St Joseph, MI, USA). For peramine analysis, the powdered samples were extracted with a methanol : water (4 : 1 v/v) mixture and the extract washed with hexane five times. The hexane fractions were discarded. Using an HPLC, separation of peramine was performed on a C18/cation exchange column (150 mm ! 4·6 mm with 5 μm beads, Alltech Associates, http://www.alltechweb.com, part number 72574). The elution programme was performed with 5% solvent A and 95% solvent B for the first 9·5 min, then a linear change to 35% A and 65% B over the next 22 min and then back to 5% A and 95% B over 0·5 min and held for 2 min. Solvent A was acetonitrile : 0·1 ammonium acetate (4 : 1, v/v) and solvent B was acetonitrile : water (9 : 1, v/v). The flow was 1·8 mL min ^{1}. Peramine retention was approximately 25 min and was detected at 280 nm. The calibration was done with an authentic chemical standard.

Lolitrem B analyses were conducted to test the viability of the *N. lolii* used in the experiment. To avoid destructive sampling of the experimental plants, samples were collected from reserve greenhouse plants in April, 4 months after planting. The sheaths and blades from 20 different plants for each cultivar and infection status were pooled. Fifty milligrams of freeze dried and lyophilized material were extracted with dichloromethane : methanol (1 : 1 v/v, 7 mL, 30 min) and purified by solid phase extraction (Varian Bond Elut Si, 100 mg/1 mL, elution with dichloromethane : acetonitrile 4 : 1). The solvent was evaporated and the sample dissolved in acetonitrile : dichloromethane (2 mL/100 μL) for HPLC-MS analyses. This was performed on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) connected to a Bruker ESQUIRE-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany) in the (+)-APCI ionization mode. Chromatographic conditions: Waters Symmetry C_{18} column (150 ! 2 mm) and a flow rate of 0·3 mL min ^{1}. Mobile phase: gradient within 8 min from 50 to 90% of solvent B, then 4 min at 90% of B (solvent A: 0·05% formic acid solution in water, solvent B: 0·05% formic acid solution in acetonitrile). MS acquisitions were performed in the 'single reaction monitoring' mode (*m/z* 686·3–628·3).

Aphids and parasitoids

Aphids were counted and identified to species level on four occasions, at 2-wkly intervals (June–July 2004). Sampling times were standardized at 5 min per plot (75 plants), to ensure uniform sampling effort, and complete aphid counts. Aphids were not removed from the plants during these surveys and counts were pooled for statistical analyses. Aphid species identification followed Blackman & Eastop (2000). The three most common cereal aphids on *L. perenne* in the region are: *Sitobion avenae* Fabricius, *Rhopalosiphum padi* L. and *Metopolophium festucae* Theobald, which are all easy to identify in the field. These species are native to Europe and occur on numerous species of Gramineae (Blackman & Eastop 2000).

To detect the abundances of primary and secondary parasitoids of aphids, all aphid mummies were collected from all plants on two of the survey dates in July 2004. Each mummy was placed into individual gelatine capsules and left to emerge in the laboratory. The identification of primary parasitoids was based on Stary (1966, 1973), that of Alloxystinae on van Veen (1999) and van Veen, Belshaw & Godfray (2003), and that of the other secondary parasitoids on Graham (1969) and Fergusson (1980). All parasitoid identifications were confirmed by Dr Frank van Veen (Imperial College London, UK).

Further grass herbivores (a total of 26 beetles and bugs) and aphid predators (a total of 109 ladybirds, syrphids, lacewings, beetles, bugs and spiders) were detected during the four aphid surveys. The overall low species abundances did not allow meaningful population density analyses for these groups.

All statistical analyses were conducted using R (version 2.1.1). Linear mixed effects models with a maximum likelihood method were calculated for the main experiment with fixed factors (1) plant cultivar, (2) endophyte infection, and (3) fertilization, and all interactions between these. Block and nested sub-blocks were treated as random factors (Pinheiro & Bates 2000). Statistical analyses of peramine concentration were restricted to endophyte-infected plants only, as uninfected plants did not contain this alkaloid. Response variables were transformed when necessary to meet the assumptions of normality and homoscedasticity. Biomass measurements and number of spikelets were $\log_{10}$ transformed, number of ears and length of ears were square-root transformed. The count data for aphids and parasitoids were pooled over the four sampling dates for the aphids and over the two sampling dates for the parasitoids. The pooled counts were square-root transformed. Pearson correlations were calculated to identify correlations between the transformed response variables at the different trophic levels. Data collected in the plant growth room had plant cultivar and endophyte infection as fixed factors and block as a random factor. The

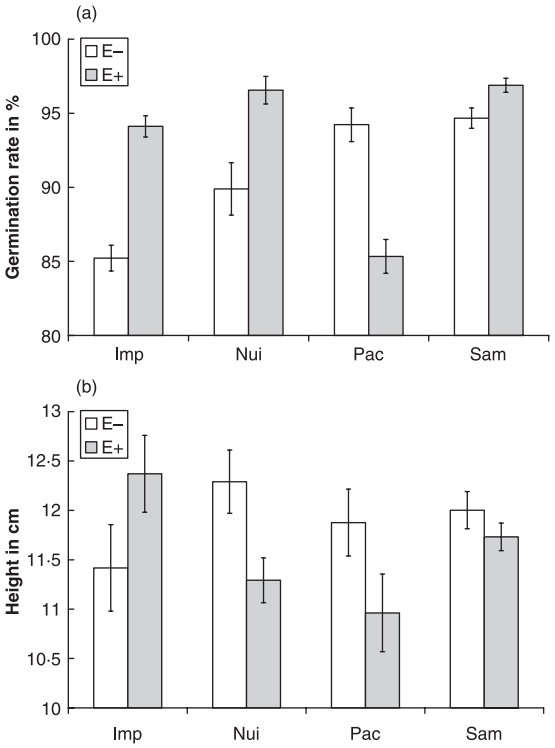


Fig. 2. Effects of endophyte infection on (a) germination rate and (b) plant height on four different cultivars of *L. perenne*. (a) Mean (± SE) germination rate in percent of the four different plant cultivars of *L. perenne* infected (E+, grey bars) and uninfected (E–, white bars) by *N. lolii* showed a significant interaction between cultivar and endophyte infection after 14 days (endophyte ! plant cultivar: $F_{3,35} = 36{\cdot}72$; $P < 0{\cdot}0001$). Endophyte infection ($F_{1,35} = 11{\cdot}59$; $P = 0{\cdot}002$) and plant cultivar ($F_{3,35} = 20{\cdot}43$; $P < 0{\cdot}0001$) were both significant. (b) Similarly, the mean (± SE) plant height in centimetres of the four cultivars after 13 days interacted with infection status (endophyte ! plant cultivar: $F_{3,35} = 4{\cdot}09$; $P = 0{\cdot}014$). Neither endophyte infection ($F_{1,35} = 1{\cdot}91$; $P = 0{\cdot}176$) nor plant cultivar ($F_{3,35} = 0{\cdot}98$; $P = 0{\cdot}414$) were significant.

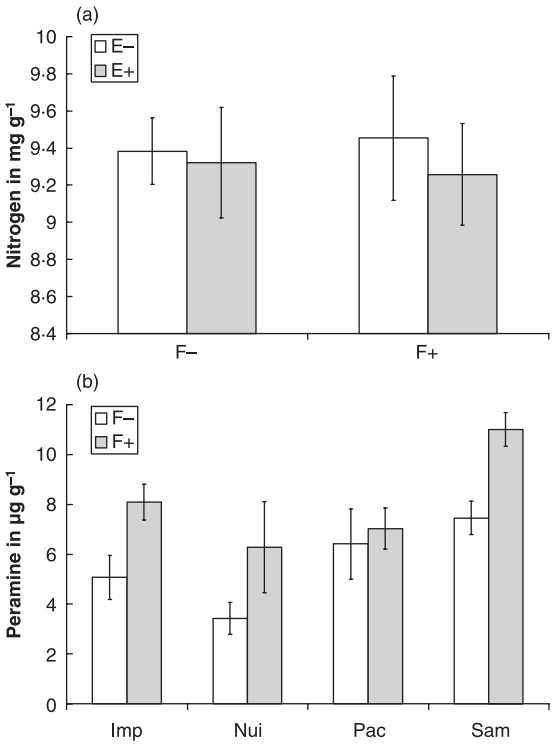


Fig. 3. (a) Effects of endophyte infection on nitrogen concentration on fertilized and not fertilized *L. perenne*. (b) Effects of fertilization on peramine concentration on four different cultivars of *L. perenne*. (a) Mean (± SE) nitrogen concentration in *Lolium perenne* above-ground tissue was not significantly affected by fertilizer addition (F+) compared with no fertilizer addition (F–), endophyte infection (E+, grey bars) compared with no infection by endophytes (E–, white bars) or the four different plant cultivars (pooled in the figure). (b) The mean (± SE) concentration of insect toxic peramine was significantly higher in fertilized *L. perenne* plants (F+, grey bars) than in not fertilized plants (F–, white bars) and significantly differed for the four plant cultivars (Imp, Nui, Pac, Sam). Note that endophyte-free plants did not contain peramine (for statistics see Table 1).

response variables germination rates and height of seedlings were not transformed. Arithmetic means and standard errors of back-transformed data are given throughout the text and shown in all figures.

Results

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There was a significant interaction between endophyte infection and plant cultivar, in terms of germination success and plant height for *L. perenne* on days 13 and 14 (Fig. 2). Eight months later, at the end of the experiment, there were no longer clear differences in plant traits between endophyte-infected and endophyte-free plants. There was a significant three-way treatment interaction (cultivar ! endophyte ! fertilizer) for total biomass and shoot biomass, as well as a significant interaction between endophyte and fertilizer for the number of ears produced (Table 1). Fertilizer addition strongly increased total above-ground biomass, shoot biomass (F– = 43·7 ± 1·5, F+ = 101·0 ± 3·7 g), ear biomass (F– = 9·7 ± 0·7, F+ = 15·4 ± 1·1 g) and number of ears (F– = 70·1 ± 5·2, F+ = 98·6 ± 6·5). These plant traits were also clearly affected by plant cultivar. For number of spikelets and ear length, plant cultivar was the only significant predictor; endophyte infection and fertilizer addition had no significant effects (Table 1).

The nitrogen concentration (Table 1, Fig. 3a), as well as carbon concentration and C : N ratios (result not shown), were not significantly influenced by any treatment. The concentration of the two alkaloids lolitrem B and peramine, which are produced by the endophyte, could only be detected in infected *L. perenne* plants. Lolitrem B concentrations were 0·41 µg g^{-1} for Imp, 0·17 µg g^{-1} for Nui, 0·16 µg g^{-1} for Pac, and 0·36 µg g^{-1} for Sam (these data could not be analysed statistically because material had to be pooled, see Materials and methods). In unfertilized plants, peramine

Table 1. Mixed effects models showing the relationship between predictor variables (plant cultivar, endophyte infection, fertilizer treatment) and plant response variables. The three biomass variables plus number of spikelets were $\log_{10}$ transformed, No. of ears per plot and ear length were square-root transformed

Predictor variables	Biomass	Shoot biomass	Ear biomass	Number of ears per plot
Cultivar (C)	$F_{3,75} = 6{\cdot}26$	$F_{3,75} = 4{\cdot}15$	$F_{3,75} = 8{\cdot}70$	$F_{3,75} = 35{\cdot}82$
	$P = 0{\cdot}0008$	**$P = 0{\cdot}009$**	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**
Endophyte infection (E)	$F_{1,75} = 0{\cdot}19$	$F_{1,75} = 0{\cdot}13$	$F_{1,75} = 0{\cdot}02$	$F_{1,75} = 0{\cdot}08$
	$P = 0{\cdot}663$	$P = 0{\cdot}724$	$P = 0{\cdot}883$	$P = 0{\cdot}781$
Fertilization (F)	$F_{1,75} = 450{\cdot}71$	$F_{1,75} = 571{\cdot}73$	$F_{1,75} = 21{\cdot}89$	$F_{1,75} = 38{\cdot}00$
	$P < 0{\cdot}0001$	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**	**$P < 0{\cdot}0001$**
C ! E	$F_{3,75} = 0{\cdot}30$	$F_{3,75} = 0{\cdot}19$	$F_{3,75} = 0{\cdot}78$	$F_{3,75} = 0{\cdot}65$
	$P = 0{\cdot}827$	$P = 0{\cdot}910$	$P = 0{\cdot}509$	$P = 0{\cdot}584$
C ! F	$F_{3,75} = 0{\cdot}26$	$F_{3,75} = 0{\cdot}32$	$F_{3,75} = 0{\cdot}43$	$F_{3,75} = 0{\cdot}74$
	$P = 0{\cdot}857$	$P = 0{\cdot}809$	$P = 0{\cdot}730$	$P = 0{\cdot}532$
E ! F	$F_{1,75} = 1{\cdot}43$	$F_{1,75} = 0{\cdot}87$	$F_{1,75} = 2{\cdot}05$	$F_{1,75} = 5{\cdot}21$
	$P = 0{\cdot}235$	$P = 0{\cdot}354$	$P = 0{\cdot}156$	**$P = 0{\cdot}025$**
C ! E ! F	$F_{3,75} = 3{\cdot}12$	$F_{3,75} = 3{\cdot}09$	$F_{3,75} = 1{\cdot}90$	$F_{3,75} = 2{\cdot}34$
	$P = 0{\cdot}031$	**$P = 0{\cdot}032$**	$P = 0{\cdot}137$	$P = 0{\cdot}081$

Predictor variables	No. of spikelets	Ear length	Nitrogen	Peramine
Cultivar (C)	$F_{3,75} = 7{\cdot}48$	$F_{3,75} = 23{\cdot}77$	$F_{3,75} = 1{\cdot}45$	$F_{3,32} = 7{\cdot}30$
	$P = 0{\cdot}0002$	**$P < 0{\cdot}0001$**	$P = 0{\cdot}235$	**$P = 0{\cdot}0007$**
Endophyte infection (E)	$F_{1,75} = 0{\cdot}00$	$F_{1,75} = 0{\cdot}25$	$F_{1,75} = 0{\cdot}33$	
	$P = 0{\cdot}954$	$P = 0{\cdot}622$	$P = 0{\cdot}569$	
Fertilization (F)	$F_{1,75} = 1{\cdot}85$	$F_{1,75} = 0{\cdot}51$	$F_{1,75} = 0{\cdot}00$	$F_{1,32} = 15{\cdot}76$
	$P = 0{\cdot}178$	$P = 0{\cdot}478$	$P = 0{\cdot}985$	**$P = 0{\cdot}0004$**
C ! E	$F_{3,75} = 0{\cdot}38$	$F_{3,75} = 2{\cdot}21$	$F_{3,75} = 0{\cdot}88$	
	$P = 0{\cdot}769$	$P = 0{\cdot}093$	$P = 0{\cdot}454$	
C ! F	$F_{3,75} = 1{\cdot}85$	$F_{3,75} = 1{\cdot}76$	$F_{3,75} = 0{\cdot}40$	$F_{3,32} = 0{\cdot}96$
	$P = 0{\cdot}146$	$P = 0{\cdot}163$	$P = 0{\cdot}757$	$P = 0{\cdot}426$
E ! F	$F_{1,75} = 1{\cdot}24$	$F_{1,75} = 1{\cdot}03$	$F_{1,75} = 0{\cdot}09$	
	$P = 0{\cdot}269$	$P = 0{\cdot}313$	$P = 0{\cdot}769$	
C ! E ! F	$F_{3,75} = 0{\cdot}26$	$F_{3,75} = 2{\cdot}31$	$F_{3,75} = 0{\cdot}59$	
	$P = 0{\cdot}855$	$P = 0{\cdot}083$	$P = 0{\cdot}625$	

Significant *P*-values are presented in bold.

concentrations were 5·07 ± 0·88 μg g^{-1} for Imp, 3·43 ± 0·64 μg g^{-1} for Nui, 6·41 ± 1·41 μg g^{-1} for Pac and 7·46 ± 0·67 μg g^{-1} for Sam, indicating a strong cultivar effect on peramine production (Table 1). Fertilizer addition significantly increased peramine concentrations in all cultivars except Pac (Table 1, Fig. 3b).

The total number of aphids summed over the four survey dates was 13 182 individuals, with *Sitobion avenae* (9487 individuals), *Rhopalosiphum padi* (2530) and *Metopolophium festucae* (1131) the three most abundant species. The abundances of *S. avenae* (F– = 61·3 ± 7·4, F+ = 136·3 ± 11·1) and *M. festucae* (F– = 5·5 ± 0·5, F+ = 18·1 ± 2·9) were increased by fertilizer treatment and *S. avenae* was also affected by plant cultivar (Table 2). There was a significant interaction between fertilizer ! endophyte treatments on the abundance of *R. padi*; numbers were greater on endophyte-infected, unfertilized plants (F–/E– = 19·2 ± 2·6, F–/E+ = 37·4 ± 6·2, F+/E– = 25·1 ± 3·9, F+/E+ = 23·7 ± 3·2), but effects were only just statistically significant (Table 2).

The number of parasitoids emerging from the collected mummies was 212 for primary parasitoids and 227 for secondary parasitoids, six species were identified as primary parasitoids and nine species as secondary parasitoids. The most abundant primary parasitoids were *Aphidius rhopalosiphi* (182 individuals), followed by *A. picipes* (13), *A. ervi* (12), *Ephedrus plagiator* (three), *Praon volucre* (one) and *Aphelinus* sp. (one). The most abundant secondary parasitoids were *Dendrocerus aphidium* (62), followed by *Asaphes suspensus* (41), *D. carpenteri* (39), *Asaphes vulgaris* (37), *Phaenoglyphis villosa* (20), *Alloxysta victrix* (15), *Coruna clavata* (nine), *Syrphophagus aphidivorus* (two) and *Alloxysta tscheki* (two). Individual numbers of *A. rhopalosiphi* were increased by fertilizer treatment (individuals per plot: F– = 1·1 ± 0·2, F+ = 2·6 ± 0·3), whereas there was no significant treatment effect on the number of *D. aphidium* (Table 2). All other species occurred at densities which were too low for population density analyses.

The total number of aphids (F– = 95·6 ± 8·7, F+ = 179·0 ± 12·8) and both primary (F– = 1·4 ± 0·2, F+ = 3·0 ± 0·3) and secondary parasitoids (F– = 1·6 ± 0·2, F+ = 3·1 ± 0·3) were enhanced by fertilizer addition to *L. perenne* (Fig. 4, Table 2). The effect of plant cultivar on total number of aphids was slightly above the

Table 2. Mixed effects models showing the relationship between predictor variables (plant cultivar, endophyte infection, fertilizer treatment) and insect response variables. Aphids, primary and secondary parasitoids each include all species of their trophic level and are pooled over all sampling dates. All response variables were square-root transformed

Predictor variables	Aphids	*Sitobion avenae*	*Rhopalosiphum padi*	*Metopolophium festucae*
Cultivar (C)	$F_{3,75} = 2{\cdot}67$	$F_{3,75} = 2{\cdot}85$	$F_{3,75} = 0{\cdot}78$	$F_{3,75} = 0{\cdot}58$
	$P = 0{\cdot}054$	$\mathbf{P = 0{\cdot}043}$	$P = 0{\cdot}511$	$P = 0{\cdot}626$
Endophyte infection (E)	$F_{1,75} = 0{\cdot}88$	$F_{1,75} = 0{\cdot}32$	$F_{1,75} = 3{\cdot}99$	$F_{1,75} = 0{\cdot}44$
	$P = 0{\cdot}352$	$P = 0{\cdot}575$	$\mathbf{P = 0{\cdot}049}$	$P = 0{\cdot}508$
Fertilization (F)	$F_{1,75} = 31{\cdot}45$	$F_{1,75} = 35{\cdot}78$	$F_{1,75} = 0{\cdot}59$	$F_{1,75} = 27{\cdot}02$
	$\mathbf{P < 0{\cdot}0001}$	$\mathbf{P < 0{\cdot}0001}$	$P = 0{\cdot}447$	$\mathbf{P < 0{\cdot}0001}$
C ! E	$F_{3,75} = 0{\cdot}11$	$F_{3,75} = 0{\cdot}19$	$F_{3,75} = 0{\cdot}08$	$F_{3,75} = 0{\cdot}69$
	$P = 0{\cdot}954$	$P = 0{\cdot}905$	$P = 0{\cdot}970$	$P = 0{\cdot}563$
C ! F	$F_{3,75} = 0{\cdot}49$	$F_{3,75} = 0{\cdot}40$	$F_{3,75} = 1{\cdot}04$	$F_{3,75} = 0{\cdot}30$
	$P = 0{\cdot}747$	$P = 0{\cdot}750$	$P = 0{\cdot}381$	$P = 0{\cdot}827$
E ! F	$F_{1,75} = 1{\cdot}65$	$F_{1,75} = 0{\cdot}39$	$F_{1,75} = 4{\cdot}20$	$F_{1,75} = 0{\cdot}02$
	$P = 0{\cdot}203$	$P = 0{\cdot}534$	$\mathbf{P = 0{\cdot}044}$	$P = 0{\cdot}892$
C ! E ! F	$F_{3,75} = 0{\cdot}61$	$F_{3,75} = 0{\cdot}96$	$F_{3,75} = 0{\cdot}25$	$F_{3,75} = 0{\cdot}67$
	$P = 0{\cdot}612$	$P = 0{\cdot}418$	$P = 0{\cdot}861$	$P = 0{\cdot}573$

Predictor variables	Primary parasitoids	*Aphidius rhopalosiphi*	Secondary parasitoids	*Dendrocerus aphidium*
Cultivar (C)	$F_{3,75} = 0{\cdot}93$	$F_{3,75} = 0{\cdot}46$	$F_{3,75} = 2{\cdot}16$	$F_{3,75} = 0{\cdot}47$
	$P = 0{\cdot}433$	$P = 0{\cdot}711$	$P = 0{\cdot}099$	$P = 0{\cdot}702$
Endophyte infection (E)	$F_{1,75} = 0{\cdot}37$	$F_{1,75} = 0{\cdot}61$	$F_{1,75} = 0{\cdot}00$	$F_{1,75} = 0{\cdot}73$
	$P = 0{\cdot}548$	$P = 0{\cdot}436$	$P = 0{\cdot}963$	$P = 0{\cdot}396$
Fertilization (F)	$F_{1,75} = 14{\cdot}34$	$F_{1,75} = 14{\cdot}36$	$F_{1,75} = 14{\cdot}86$	$F_{1,75} = 1{\cdot}66$
	$\mathbf{P = 0{\cdot}0003}$	$\mathbf{P = 0{\cdot}0003}$	$\mathbf{P = 0{\cdot}0002}$	$P = 0{\cdot}202$
C ! E	$F_{3,75} = 1{\cdot}85$	$F_{3,75} = 1{\cdot}92$	$F_{3,75} = 0{\cdot}11$	$F_{3,75} = 0{\cdot}55$
	$P = 0{\cdot}145$	$P = 0{\cdot}133$	$P = 0{\cdot}952$	$P = 0{\cdot}648$
C ! F	$F_{3,75} = 0{\cdot}67$	$F_{3,75} = 1{\cdot}02$	$F_{3,75} = 1{\cdot}58$	$F_{3,75} = 0{\cdot}39$
	$P = 0{\cdot}574$	$P = 0{\cdot}387$	$P = 0{\cdot}202$	$P = 0{\cdot}758$
E ! F	$F_{1,75} = 0{\cdot}01$	$F_{1,75} = 0{\cdot}01$	$F_{1,75} = 0{\cdot}13$	$F_{1,75} = 3{\cdot}42$
	$P = 0{\cdot}916$	$P = 0{\cdot}931$	$P = 0{\cdot}723$	$P = 0{\cdot}068$
C ! E ! F	$F_{3,75} = 0{\cdot}40$	$F_{3,75} = 0{\cdot}41$	$F_{3,75} = 0{\cdot}45$	$F_{3,75} = 0{\cdot}78$
	$P = 0{\cdot}757$	$P = 0{\cdot}748$	$P = 0{\cdot}715$	$P = 0{\cdot}507$

Significant *P*-values are presented in bold.

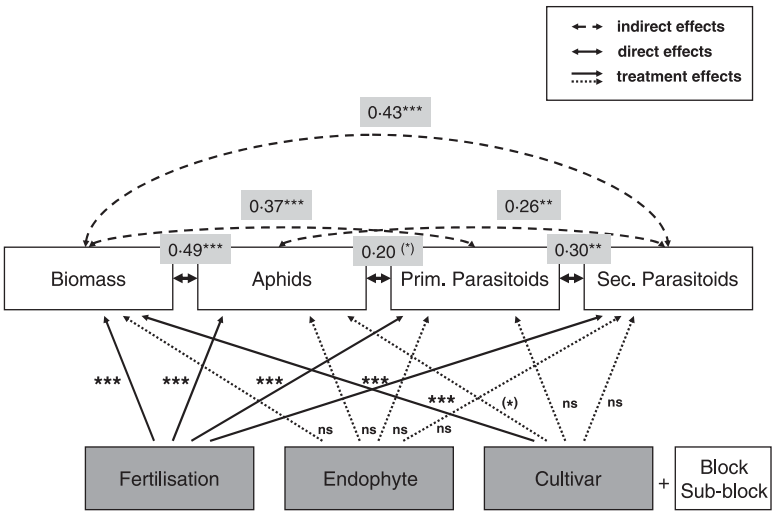

Fig. 4. Summary of the mixed effects models for the main treatment effects and Pearson correlations between the transformed response variables (log_{10}: biomass and square root: aphids, primary (Prim.) and secondary (Sec.) parasitoids). Fertilization increased plant biomass, and aphid and parasitoid abundance significantly. ***$P < 0{\cdot}001$; **$P < 0{\cdot}01$; (*)$P = 0{\cdot}05$–$0{\cdot}06$; NS, P# 0·1 (for statistics see Table 2 and Material and methods). Footnote: between predictor variables and response variables solid lines show significant relations, dotted lines show not significant relations..

significance level, whereas endophyte infection had no negative effect on the abundances of species at the three trophic levels, even though the insect-toxic peramine occurred only in plants with the fungal endophyte (Table 2; Fig. 3b). Plant biomass and the numbers of aphids, primary and secondary parasitoids were all positively correlated (Fig. 4), indicating that the effects of increased resource availability through fertilizer addition moves up the food chain. Therefore, it is not surprising that fertilizer addition had strong positive effects on all trophic levels in this insect food web. To remove the direct (plant biomass) effect of fertilizer, aphid and parasitoid numbers were divided by plant biomass. These biomass-corrected densities showed a significant plant cultivar effect on aphids ($F_{3,75} = 6{\cdot}06$, $P = 0{\cdot}0009$); all other predictors for aphid and parasitoid numbers were not significant (all $P > 0{\cdot}1$).

Discussion

In our fully factorial field experiment, fertilizer addition strongly enhanced the abundances of naturally colonizing aphids and parasitoids on agricultural

grasses. Plant cultivar had a small effect on insect species abundance, while endophyte infection of the resource plant had no negative effect on insect abundances in this study. The absence of an effect of endophyte infection is in contrast to short-term laboratory trials. For example, clear negative effects of the endophyte *N. lolii* have been shown for herbivores and predators associated with *L. perenne* (Meister *et al.* 2006; de Sassi *et al.* 2006).

The aphid *R. padi* is known to be negatively affected by the presence of *N. coenophialum* in Tall Fescue (*L. arundinacea*), with the associated insect-toxic loline group of compounds (Davidson & Potter 1995; Hunt & Newman 2005), and by *N. lolii* and associated peramine production in *L. perenne* (Meister *et al.* 2006). It is surprising, therefore, that there was a trend towards higher densities of this aphid species on infected unfertilized plants, compared with uninfected and fertilized plants in our experiment. In another field experiment conducted in 2005, *R. padi* was also more abundant on infected *L. perenne* (Jochen Krauss, unpublished data). We currently have no explanation for why this aphid species shows such contrasting results. Endophyte effects on the aphids *M. festucae* and *S. avenae* could not be detected in our study; this supports data from laboratory studies that show that *M. festucae* has no clear negative response to endophyte infection (Simone Härri, unpublished data). *Sitobion avenae* colonizes ears of the grass and therefore depends on ear rather than leaf quality (Honek 1991). The concentration of peramine in ears is, however, unknown and was not measured separately in our study. Ear biomass, number of ears, ear length and number of spikelets all differed between plant cultivars; cultivar also significantly affected the abundance of *S. avenae*. In the absence of a fertilizer-related increase in foliar nitrogen concentration, the increase in abundance of *S. avenae* and *M. festucae* is likely to be linked to the overall increase in above-ground plant biomass following fertilizer treatment. Growth dilution of foliar N concentrations is a common phenomenon (Johnson, Ball & Walker 1997) and is likely to explain the lack of concentration increase observed in this study.

The increase in parasitoid numbers associated with fertilizer treatment appears to be a direct result of increased aphid availability resulting from the treatment-related increase in plant biomass. Correlations between the four trophic levels – plants, aphids, primary and secondary parasitoids – make this interpretation plausible. Such cascading trophic interactions are common in food webs and have frequently been described for terrestrial webs (e.g. Schmitz 1993; Dyer & Stireman 2003).

Endophyte infection did not provide any significant defence against the aphid herbivores in our study. Similarly, neither the treatment-related increase in peramine production nor the effect of plant cultivar affected the level of herbivore protection offered by the endophyte. Elsewhere, a further peramine producing *Neotyphodium* species has also been shown to provide no protection against a grasshopper species feeding on infected Arizona Fescue (Saikkonen *et al.* 1999). The relatively small effects of endophytes on aphids and parasitoids in our study might be explained by the relatively low concentrations of peramine found in our *L. perenne* plants (unfertilized: 5·5 $\mu g\ g^{-1}$, fertilized 8·0 $\mu g\ g^{-1}$). Other studies have reported concentrations in excess of 10·0 $\mu g\ g^{-1}$ (e.g. Ball *et al.* 1995; Spiering *et al.* 2002), which is also the threshold level for feeding deterrence for the Argentine Stem Weevil (Keogh *et al.* 1996). Peramine concentrations below 3·0 $\mu g\ g^{-1}$ are generally considered nontoxic for invertebrate herbivores (Siegel & Bush 1996).

Another reason for the relatively small effect of endophytes on insect herbivores and their parasitoids might be as a result of the experiment being conducted under field conditions, with numerous indirect interactions between species and insects having a wide choice of plants on which to feed and oviposit, in contrast to more controlled laboratory conditions. Furthermore, the clear effect of endophytes on plant performance in our 2-week growth room experiment disappeared 8 months later under field conditions. The main driver for plant performance in the field was fertilization and, to a lesser degree, plant cultivar. In the growth room study, endophyte infection and plant cultivar showed significant interactions in terms of germination success and plant height. These findings are consistent with other laboratory studies where plant performance is often affected by interactions between endophyte infection and host-plant genotype (Cheplick 1998, 2004; Cheplick & Cho 2003).

In conclusion, our study showed that under field conditions endophyte effects on plant performance, herbivores and natural enemies are less consistent than laboratory studies suggest. For aphid populations and their parasitoids, fertilizer addition at agricultural rates has much stronger effects on abundance than endophyte and alkaloid presence. The increase in peramine concentrations associated with fertilizer addition was not sufficient to decrease aphid population sizes. Overall, in this study system with four endophyte-infected agronomic grass cultivars and trophic interactions based on aphids and their parasitoids, we found that the effect of fertilizer on aphid and parasitoid abundance was greater than the effect of plant cultivar on *L. perenne*, and the effect of endophyte infection by *N. lolii* was minimal.

Acknowledgements

We thank Thomas Bultman, Stan Faeth and Dan Hare for helpful comments and Adrian Leuchtmann and Brian Tapper for their grass and endophyte information. Frank van Veen kindly helped with the identification of the aphid parasitoids. We thank Urs Stalder, Claudio de Sassi, Susanne Müller, Julia Nüscheler and Tobias Züst for laboratory and field assistance and Barbara Meister for pilot studies. The project was funded by a grant of the Swiss National Science Foundation to Christine Müller (Grant number 631-065950).

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Received 20 June 2006; revised 25 August 2006; accepted 15 September 2006
Editor: J. Daniel Hare

ACKNOWLEDGMENTS

“They danced down the streets like dingledodies, and I shambled after as I've been doing all my life after people who interest me, because the only people for me are the mad ones, the ones who are mad to live, mad to talk, mad to be saved, desirous of everything at the same time, the ones who never yawn or say a commonplace thing, but burn, burn, burn like fabulous yellow roman candles exploding like spiders across the stars and in the middle you see the blue centerlight pop and everybody goes ‘Awww!’”

JACK KEROUAC

ACKNOWLEDGMENTS

The first and by far biggest thanks goes to Christine Müller who was much more than a supervisor. I could always count on her, not only in science related things. There is probably no other supervisor that would have given me so much freedom. Her belief in my abilities and her immense support made this thesis possible and the time in Zürich such an unforgettable experience. I think we are a great team and I hope we will be able to continue our team work in as many collaborations as possible. With your admirable strength and power, you will always be my role model!

An enormous thanks goes to the rest of the Müller group. Such a wonderful and nerdy crowd! The group was much more than a working team, it was more like a family to me. Jochen Krauss, "my" post-doc, thanks for everything! Especially for always backing me up against any one else and for being the grumpy German I liked very much. Dennis Hansen, what would I have done without you? Thanks for so much, for your immense support, for sharing the passion for Jack Bauer, for making me laugh so many times, for your genius personification of Bernard Black, for providing me with chocolate whenever I needed it, for listening to my ramblings about aphids and endophytes and life in general, but especially for being such a wonderful friend. Chris Kaiser, thanks for introducing me to Doppelkopf, for offering me wonderful German white wines, for cooking for me and keeping me healthy, and for many inspiring discussions about work, universe and life. Nancy Bunbury, I add you here as honorary group member, whenever I was invited to you and Chris, it just felt like home. You don't know how much I enjoyed these evenings and the feeling of having a second home. Thanks also for introducing me to so many wonderful books and for your last minute corrections!

A special thank goes to Dr. Tom Jones for being the only one who understands (or at least pretends) my complicated scientific thoughts and for always being ready to have a beer or two. Matthias Albrecht, thanks for patiently listening to all my daily stories about wedding cards of friends and all other kind of weird unimportant stuff. Thanks Simone von Burg for continuing the aphid work and I wish you good luck with all scientific and political issues that might pop up during your PhD. Atlant Bieri and Theresa Karpati, thank you two for making my first steps as a supervisor so easy and entertaining. Thanks to Tobias Züst for his immense passion for the world of aphids and endophytes and for many good ideas and for a lot of practical help. Julia Nüscheler, obrigada, for everything! Counting aphids was much more fun with you than with anyone else. Keep your open mind and continue to live your life. Thanks also very much for inviting me to Brazil.

Daniel Trujillo, you did a great job with my aphids and parasitoids and I think you are a real enrichment for our group. Hope you can play Frisbee soon again. Thanks very much Barbara, Claudio, Gabor, Cozzi, Karin, Céline and Nicolas for making the group so special. Susanne Müller, I don't know what I would have done without all your help in the lab! I was impressed by how you fought your way through all the dirty PET bottles and plant pots every week. It was also good to know that I can leave for holidays and everything is still running when I am back. Thanks so much for that! Thanks also to Virginie Boreux for helping me with experiments and especially for cutting and gluing all the PET bottles.

Apart from Christine's group, Alex Fergus and Chris-P-Philipson were particularly important for me during these years at the Ifu and I hope they will also be in the future. Alex, your chaotic Kiwi mind is truly amazing. Thanks for dragging me out of the office whenever I was close to getting mental. ChrisP, I was really sad when you left the office and I missed having you opposite me every day. A special thanks goes to Eva Vojtech. You made me feel that there is always someone to talk to. Jana Petermann, thanks for sharing the climbing passion and for many inspiring late evening discussions. Thanks also to Steffi, Luca, Sophie and Dominik. A big thanks goes to Bernhard Schmid for doing such an amazing job in leading this institute. Thanks to Andy Hector and Lindsay Turnbull for always helping me with statistics and other aspects of science. Thanks to René Husi, Theres Zwimpfer, Alex Schwendener and Petra Lindemann-Matthies for all the practical help. Isabel Schöchli, you are the angel of this institute!

I also want to thank Marta Manser, Lukas Keller and Christine for giving me the opportunity to assist in the field course every year. You always give me the impression that you appreciate and trust my opinion. Thanks also to Jonathan Newman for offering me a post-doc position, for extremely inspiring discussions, and for making me feel like a true scientist.

Andrea, it was great to get to know you. I will miss our coffee breaks and all our adventures. The mountains are best conquered with you, Jens and Andreas! I always had the feeling that it is really special to be such a good team and I hope we will be able to go to many more adventures together. Daniel Rankin, I will never forget our nightlong discussions about life, books and women. A special thanks to Uli for a wonderful time, for introducing me to climbing, and for many stimulating scientific discussions. Annette, thanks for being such a good discussion partner. I really like our lively bouncing back and

forth discussions. Sala, thanks for not disappearing completely out of my life. I appreciate it very much.

A big thanks to all my climbing buddies but especially to Mirjam. Thanks also to Katja, Viola, James, Niels, Iso and Pete for sharing a little part of your life with me. Getting to know wonderful persons and spend time with them is the real inspiration of my life. Paquita, thank you so much for everything but particularly for sharing some of your positive energy with me. I really hope we stay in contact and I am looking forward to visit you in Africa when you have found your dream job. I am sure you will continue to live your dreams. Fabienne, I feel honored to have you as a friend for already such a long time. You always made me realise that there is a life outside science and your immense support in whatever aspect of my life is just priceless. Thanks very much for everything! And of course, immense big thanks to Fränzi for a friendship that does not need much words.

Thanks to my PhD committee, Stan Faeth, Charles Godfray, Andy Hector and Adrian Leuchtmann. Stan, Keith Clay and Kari Saikkonen for offering to review my thesis. I really appreciate it! A big thanks to the whole endophyte community, but especially to Stan, Keith and Kari, for making my start into the scientific life so easy and inspiring. I was amazed how easy it was to approach this “big names”.

Last but not least a big thanks to my family. Especially my mum provides me with so much mental support for which I cannot thank her enough. She is always there for me and she understands me without words. And a special thanks to my Dad, Rolf. And finally, the Dodo, the Kiwi and Jones the Gecko for keeping me company during all the late night hours I spent in my home – office.

CURRICULUM VITAE

"I may not have gone where I intended to go, but I think I have ended up where I needed to be."

DOUGLAS ADAMS

CURRICULUM VITAE

SURNAME	Härri
FIRST NAMES	Simone Anne
DATE OF BIRTH	July 31, 1976
HOMETOWN	Birrwil (AG)

EDUCATION

Gymnasium	Kantonsschule Zofingen, Matura-Typus D, 1992 – 1996
University	University of Basel, 1996 - 2002 Main subject: Population biology Secondary subjects: Vertebrate biology, developmental biology and plant ecology Minor subject: Business management
Diploma thesis	*"Population dynamics and the effects of habitat factors on occupancy in an aphid island metapopulation"* University of Basel, Ecological Institute Jena, Germany and Tvärminne Zoological Station, Finland Supervised by Prof. W. W. Weisser
Dissertation	*"Fungal endosymbionts of grasses, and their effects on multitrophic interactions"* Institute of Environmental Sciences, University of Zürich November 2003 – October 2007 Supervised by Prof. C. B. Müller

PEER-REVIEWED PUBLICATIONS

HÄRRI, S.A., KRAUSS, J & MÜLLER, C.B. (accepted) Natural enemies act faster than endophytic fungi in population control of cereal aphids. *Journal of Animal Ecology*.

ZÜST, T., **HÄRRI, S.A.** & MÜLLER, C.B. (accepted) Endophytic fungi decrease available resources for the aphid *Rhopalosiphum padi* and impair their ability to induce defences against predators. *Ecological Entomology*.

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